Molecular Encapsulation: Cyclodextrin-Based Analogues of Heme-Containing Proteins

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Abstract: The active site heme prosthetic group of such species as hemoglobin, the cytochrome P-450s, and the peroxidases is enveloped within a protective hydrophobic environment located adjacent to a binding site for exogeneous compounds. In addition to providing these physical attributes, the protein component also acts as a carrier, transporting the heme to the appropriate cellular environment. We have replaced this proteinoid appendage with a cyclodextrin-based protective sheath to create artificial analogues of heme-containing proteins. Encapsulated within this saccharide-coated barrier, the heme moiety exhibits many of the characteristics typically reserved for its naturally occurring protein-based counterparts.

Introduction

Heme-containing proteins participate in a range of activities, including oxygen transport and activation.[1] The proteinoid appendage in these species functions (i) as a steric impediment, precluding deleterious porphyrin–porphyrin interactions, (ii) as an environmental determinant, rendering the milieu that envelops the heme moiety hydrophobic, and (iii) as a molecular vehicle, transporting the heme to the appropriate cellular environment. The construction of synthetic species capable of mimicking the activities of heme-containing proteins has been a long, sought-after goal.[2] Such studies have not only served to expand our knowledge of the chemical basis of activity exhibited by this remarkable class of proteins but also may have noteworthy implications in medicine as well (e.g., blood substitutes).[3] We report herein that the molecular encapsulation of both porphyrins and metalloporphyrins by cyclodextrins provides inclusion complexes that exhibit many of the same properties which characterize such species as the oxygen-binding proteins and the cytochrome P-450s.

Experimental Section

Materials and Instrumentation. ^{1}H NMR and ^{13}C NMR were obtained at 300 and 22.5 MHz, respectively, on a Varian Gemini-300 spectrometer except for nuclear Overhauser experiments which were performed on a Varian VXR-400S spectrometer at 20 °C. Chemical shifts are reported with respect to tetramethylsilane. Infrared spectra were recorded on a Beckman Acculab 4. UV–visible measurements were performed on a Perkin Elmer Model Lambda 2 spectrophotometer employing 1-cm polystyrene cells. Constant temperature was maintained via a circulating bath. Mass spectral analysis was performed on a VG-70SE mass spectrometer. All solvents were distilled prior to use. Acetone and chloroform were distilled from sodium sulfate. Methylene chloride was distilled from P_2O_5. All purchased reagents were employed without further purification.

Alkylation of 4-Hydroxybenzaldehyde with *N*-(3-bromopropyl)-phthalimide (Preparation of Compound 1). The following is based on a procedure described by Lever et al.:[4] 4-hydroxybenzaldehyde (1.50 g, 12.3 mmol), anhydrous K_2CO_3 (2.20 g, 15.9 mmol), (3-bromopropyl)-phthalimide (3.30 g, 12.3 mmol), and DMF (15.0 mL) were introduced into an oven-dried, 25-mL, round-bottomed flask (magnetic stirring bar, reflux condenser, Ar atmosphere). The solution was heated to 65 °C for 4 h and then stirred at room temperature for 18 h. The solvent was removed under reduced pressure, and the residue was taken up in $CHCl_3$. The organic layer was washed with 5% aqueous NaOH, distilled water, and brine and then dried over K_2CO_3. Upon removal of the solvent, a yellow solid was obtained which was recrystallized from ethyl acetate to provide 3.22 g (84.7% yield) of white needles: mp 127–128 °C; ^{1}H NMR (CDCl$_3$) δ 9.76 (s, 1 H, CHO), δ 7.67 (m, 6 H, phthalimide, phenyl), δ 6.79 (q, 2 H, phenyl), δ 4.03 (t, 2 H, OCH$_2$), δ 3.84 (t, 2 H, NCH$_2$), δ 2.14 (p, 2 H, CH$_2$CH$_2$CH$_2$); ^{13}C NMR (CDCl$_3$) δ 191.6 (aldehyde carbonyl), 169.1 (imide carbonyl), 164.4, 134.6, 132.7, 132.5, 130.6, 123.9, 115.2, 66.4 (OCH$_2$), 35.5 (NCH$_2$), 28.4 (CH$_2$CH$_2$CH$_2$); IR (film) 1762, 1700, 1680, 1250, 835 cm^{-1}; mass spectrum, *m/e* calculated for

$C_{18}H_{15}NO_4$ = 309.27, found 309.27 (M$^+$), 188.20, 160.16, 130.13, 77.11, 41.08.

Preparation of the 5,10,15,20-Tetraphthalimidoporphyrin 2. The aromatic aldehyde 1 (2.0 g, 6.40 mmol) and pyrrole (433 mg, 6.4 mmol) were condensed according to the method of Lindsey.[5] After removal of the solvent, the purple residue was dissolved in CHCl$_3$, and the organic layer was then extracted with 5% NaOH. The organic layer was subsequently dried over Na$_2$SO$_4$ and then removed in vacuo. The residue was subjected to rapid column chromatography (silica gel) with CHCl$_3$ as the eluent to remove the tarry byproducts. A dark purple band was collected, and the solvent evaporated. The resultant solid was once again chromatographed (silica gel; 60:40 ethyl acetate/hexane). After an intense green band had passed through the column, the desired porphyrin was eluted off the column with CHCl$_3$ to provide 210 mg (9.2% yield) of porphyrin 2: ^{1}H NMR (CDCl$_3$) δ 8.85 (s, 8 H, pyrrole), δ 8.1–7.9 (two doublets, 16 H, phthalimide), δ 7.85 (d, 8 H, ortho phenyl hydrogens), δ 7.20 (d, 8 H, meta phenyl hydrogens), δ 4.38 (t, 8 H, OCH$_2$), δ 4.12 (t, 8 H, NCH$_2$), δ 2.40 (m, 8 H, CH$_2$CH$_2$CH$_2$), δ –2.80 (s, 2 H, NH); ^{13}C NMR (CDCl$_3$) 169.2 (imide carbonyl), 159.2, 148.0, 136.2, 135.4, 134.5, 132.9, 131.5, 123.0, 120.3, 113.3, 66.4 (OCH$_2$), 35.9 (NCH$_2$), 28.7 (CH$_2$CH$_2$CH$_2$); mass spectrum, calculated for $C_{88}H_{66}N_8O_{12}$ = 1427.48, found = 1427.58 (M$^+$); UV–vis (CHCl$_3$) 650, 594, 556, 519, 422 nm.

Preparation of the 5,10,15,20-Tetraaminoporphyrin 3. The 5,10,15,20-tetraphthalimidoporphyrin 2 (0.100 g, 0.07 mmol) and THF (25 mL) were introduced into a 100-mL, round-bottomed flask (magnetic stirring bar, reflux condenser), and the mixture was heated to 60 °C. Hydrazine (18 mL) was subsequently introduced, and the resultant mixture was heated to reflux for 5 h and then allowed to cool to room temperature. The lower colorless layer was discarded, and the upper purple THF layer was collected and evaporated under reduced pressure. The purple solid was dissolved in mineral acid (10 mL concentrated HCl/30 mL distilled water) and stirred for 1 h at room temperature. The porphyrin was precipitated from solution by addition of aqueous NaOH. The precipitate was collected by centrifugation and washed several times with water. The purple solid was then dissolved in chloroform (dissolution was assisted by the introduction of a few drops of methanol), and the organic phase was extracted with aqueous NaOH, brine, and water and then dried. Removal of the solvent provided the desired material in 85.0% yield (0.054 g): ^{1}H NMR (CDCl$_3$) δ 8.90 (s, 8 H, pyrrole), δ 8.11 (d, 8 H, ortho phenyl hydrogens), δ 7.26 (d, 8 H, meta phenyl hydrogens), δ 4.30 (t, 8 H, OCH$_2$), δ 3.07 (t, 8 H, NCH$_2$), δ 2.11 (m, 8 H, CH$_2$CH$_2$CH$_2$), δ –2.78 (s, 2 H, NH); ^{13}C NMR (D$_2$O/DCl) 164.3, 147.1, 143.8, 135.5, 130.5, 123.8, 118.9, 69.7 (OCH$_2$),

(1) *Metal Ion Activation of Dioxygen*; Spiro, T. G., Ed.; John Wiley & Sons: New York, 1980.

(2) (a) Baldwin, J. E.; Perlmutter, P. *Top. Curr. Chem.* **1984**, *121*, 181. (b) Tsuchida, E.; Nishide, H. *Top. Curr. Chem.* **1986**, *132*, 63. (c) T. C. Bruice, *Ann. N.Y. Acad. Sci.* **1986**, *471*, 83. (d) J. T. Groves, *Ann. N.Y. Acad. Sci.* **1986**, *471*, 99.

(3) (a) Tsuchida, E. *Ann. N.Y. Acad. Sci.* **1985**, *446*, 429. (b) Tsuchida, E.; Nishide, H.; Ohno, H. *Biomat. Art. Cells, Art. Org.* **1988**, *16*, 313.

(4) Lever, O. W.; Bell, L. N.; Hyman, C.; McGuire, H. M.; Ferone, R. *J. Med. Chem.* **1986**, *29*, 665.

(5) Lindsey, J. B.; Schreiman, I. C.; Hsu, H. C.; Kearney, P. C.; Marguerettaz, A. M. *J. Org. Chem.* **1987**, *52*, 827.

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41.1 (NCH_2), 30.5 ($CH_2CH_2CH_2$); mass spectrum calculated for $C_{56}H_{58}N_8O_4 = 907.39$, found = 907.39 ($M^+$); UV–vis ($CHCl_3$) 651, 593, 556, 518, 422 nm.

Insertion of Fe(III) into the 5,10,15,20-Tetraphthalimidoporphyrin 2 (Preparation of Fe(III) 5,10,15,20-Tetraphthalimidoporphyrin 4).[6] The 5,10,15,20-tetraphthalimidoporphyrin 2 (0.14 g, 0.09 mmol) was dissolved in DMF (15 mL) and heated to reflux (25-mL, round-bottomed flask, magnetic stirring bar, reflux condenser, Ar). Anhydrous ferric chloride (0.20 g, 1.23 mmol) was added to the solution, and heating was continued until the reaction was complete (loss of long wavelength UV–visible fluorescence, 2 h). The DMF was removed under reduced pressure, and the purple residue was dissolved in $CHCl_3$ and chromatographed on alumina with $CHCl_3$ as the eluent. The reddish-brown band was collected and washed three times with concentrated HCl to break up μ-oxo dimer and subsequently dried with Na_2SO_4: 96.0% yield; ^1H NMR ($CDCl_3$) δ 80.0 (β-pyrrole), δ 13.0, 11.8 (meta phenyl hydrogens), δ 8.2, 7.9 (phthalimide), δ 5.6 (ortho phenyl hydrogens), δ 4.60 (OCH_2), δ 3.00 (NCH_2), δ 2.20 ($CH_2CH_2CH_2$); mass spectrum, calculated for $C_{88}H_{64}N_8O_{12}FeCl$ minus three propylphthalimido groups and $Cl^- = 919.60$, found = 919.05; calculated for $C_{88}H_{64}N_8O_{12}FeCl$ minus four propylphthalimido groups and $Cl^- = 732.41$, found = 732.05; UV–vis (DMF) 588, 535, 418 nm.

Preparation of the Fe(III) 5,10,15,20-Tetraaminoporphyrin 5. The Fe(III) porphyrin 5 was prepared in a fashion analogous to that described for the tetraaminoporphyrin 3. However, the desired material was isolated from the reaction mixture employing the following protocol. The purple solid (obtained upon removal of the upper THF layer) was dissolved in acetic acid and subsequently precipitated with 5% NaOH (this procedure removes residual hydrazine and phthalhydrazide). The purple precipitate was collected via centrifugation, and the supernatent was discarded. This process was repeated four additional times, and then the purple solid was extensively washed with water. The solid was freeze dried to provide 5 in 67.4% yield: ^1H NMR (5 was first dissolved in ~40 μL of CD_3COOD and then $CDCl_3$ was added to give a final volume of 1 mL) δ 79.3 (β-pyrrole), δ 12.9, 11.8 (meta phenyl hydrogens), δ 5.4 (ortho phenyl hydrogens), δ 3.9 (OCH_2), δ 2.8 (NCH_2), δ 2.1 (CH_2C-H_2CH_2); mass spectrum, calculated for = $C_{56}H_{56}N_8O_4FeCl = 995.3$, found = 960.2 ($M^+ - Cl$); UV–vis ($CHCl_3$) 630, 575, 417 nm.

Heptakis(2,6-di-O-methyl)-β-cyclodextrin ("Me-CD", 9). Me-CD was synthesized employing the protocol previously described by Szejtli et al.[7]

Nuclear Overhauser Enhancement Spectroscopy (NOESY) Experiments on the Cyclodextrin–Porphyrin 3 Inclusion Complex. The samples were 8 mM in Me-CD and 8 mM in porphyrin 3 with D_2O (pD 4.0) as the solvent. The spectra were obtained with the pulse sequence $90°–t_1–90°–mix–90°–acquire(t_2)$. The 256 experiments were performed with 96 scans per experiment. Eight dummy scans were employed. The relaxation delay was 300 ms. The time domain data with a spectral width of 4625 Hz in F1 and F2 was apodized with a shifted sine-bell function, zerofilled to a data matrix of 2K × 2K, fourier transformed, and symmetrized.

Determination of Association Constants. We employed the treatment described by Connors[8] to determine the association constants affiliated with the formation of the 1:1 and 2:1 Me-CD/porphyrin complexes. To summarize briefly, eq 1 is the mathematical representation of a multiple

$$\frac{\Delta A}{G_t b} = \frac{\beta_{11}\Delta\epsilon_{11}[H] + \beta_{12}\Delta\epsilon_{12}[H]^2}{1 + \beta_{11}[H] + \beta_{12}[H]^2} \quad (1)$$

equilibria system containing two complexes (1:1 and 2:1 host/guest), where ΔA = absorbance change at a given wavelength, b = cuvette path length, G_t = total guest concentration, $[H]$ = free host concentration, β_{11} = K_{11} = $[GH]/[G][H]$, β_{12} = $K_{11}K_{12}$ (where K_{12} = $[GH_2]/[GH][G]$), and $\Delta\epsilon_{11}$ = the extinction coefficient of the 1:1 complex minus the extinction coefficients of the free ligand and free guest at a given wavelength. An initial estimate of $\beta_{11}\Delta\epsilon_{11}$ was obtained from eq 1 by plotting $\Delta A/G_t b$ versus $[H]$ and assessing the slope of the saturation curve near $[H] = 0$. A preliminary assessment of $\Delta\epsilon_{12}$ was procured by rearranging eq 1 as eq 2 and then allowing $[H]$ to approach infinity [eq 3]. The

$$\frac{\Delta A}{G_t b} = \Delta\epsilon_{12} + \frac{(\Delta\epsilon_{11} - \Delta\epsilon_{12})\beta_{11}[H] - \Delta\epsilon_{12}}{1 + \beta_{11}[H] + \beta_{12}[H]^2} \quad (2)$$

$$\frac{\Delta A}{G_t b} = \Delta\epsilon_{12} + \frac{(\Delta\epsilon_{11} - \Delta\epsilon_{12})\beta_{11}}{\beta_{12}[H]} \quad (3)$$

(6) All metal insertions were performed as described: Adler, A. D.; Longo, F. R.; Kampas, F.; Kim, J. *J. Inorg. Nucl. Chem.* **1970,** *23,* 2443.

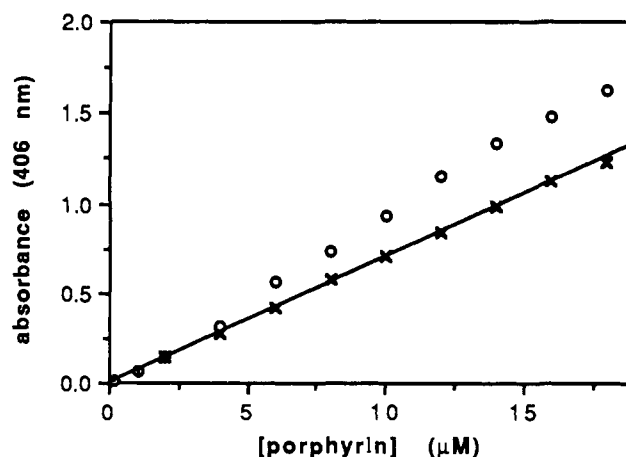(7) Szejtli, J.; Jodal, I.; Fugedi, P.; Neszmelyi, A. *Starch* **1980,** *32,* 165.

Figure 1. Absorbance (λ = 406 nm) of the tetraaminoporphyrin as a function of concentration (0.20–18.0 μM) in the absence (O) and presence (X) of Me-CD (800-fold excess maintained at all concentrations). Under the latter conditions, the amount of porphyrin present in solution as the 2:1 inclusion complex 10 varied from 90% (0.20 mM porphyrin) to 99% (18.0 μM porphyrin). Performed at pH 6.0 (50 mM succinic acid) and 25 °C. The solid line represents the expected behavior calculated from Beer's law based on the absorbance of the porphyrin at 0.20 μM.

estimates of $\beta_{11}\Delta\epsilon_{11}$ and $\Delta\epsilon_{12}$ were then employed to obtain initial values of β_{11} and β_{12} from a second rearranged form of eq 1, specifically eq 4 (note: we let $[H] = H_t$). The β_{11} and β_{12} parameters were then used to

$$\frac{1}{[H]} - \frac{\beta_{11}\Delta\epsilon_{11}G_t b}{\Delta A} = \beta_{12}[H]\left(\frac{\Delta\epsilon_{12}G_t b}{\Delta A} - 1\right) - \beta_{11} \quad (4)$$

acquire new estimates of $\beta_{11}\Delta\epsilon_{11}$ and $\Delta\epsilon_{12}$ from eq 5, a third rearranged form of eq 1. This process was repeated until stable β_{11} and β_{12} values were obtained, which were then converted into the K_{11} and K_{12} parameters.

$$\frac{\Delta A}{G_t b}(1 + \beta_{11}[H] + \beta_{12}[H]^2)[H]^{-1} = \beta_{11}\Delta\epsilon_{11} + \beta_{12}\Delta\epsilon_{12}[H] \quad (5)$$

The increase in absorbance of the porphyrin (1.0 μM) at 420 nm as a function of Me-CD concentration (5.0–500 μM) was monitored using a Perkin Elmer Model Lambda 2 spectrophotometer equipped with a thermostatted cell compartment maintained at 60 °C. All measurements on the free base porphyrin were performed with polystyrene cuvettes (1.0-cm path length). Studies on the corresponding Fe(III) porphyrin (1.0 μM) were executed at 413 nm and room temperature (~25 °C, nonthermostatted) utilizing 10.0-cm path length cylindrical quartz cells. A single stock solution of 3 (10 μM porphyrin in a 37 mM KCl/17 mM HCl buffer at pH 2.0) and two Me-CD stock solutions (1.0 mM Me-CD in 50 mM succinic acid at pH 5.0 for the experiments containing a final concentration of 5.0–125 μM Me-CD; 10.0 mM Me-CD in 50 mM succinic acid at pH 5.0 for the experiments containing a final concentration of 150–500 μM Me-CD) were employed. The order of addition of the component solutions were buffer (50 mM succinic acid at pH 5.0), Me-CD stock, and porphyrin stock. The resultant mixture was preincubated for 30 min at 60 °C prior to absorbance measurements. The Fe(III) porphyrin 5 stock solution was prepared by diluting 400 μL of a 6.25 mM Fe(III) porphyrin in glacial acetic acid to 50 mL with distilled deionized water. Two Me-CD stock solutions (2.0 mM Me-CD in 50 mM citric acid at pH 3.0 for the experiments containing a final concentration of 4.0–25 μM Me-CD; 50.0 mM Me-CD in 50 mM citric acid at pH 3.0 for the experiments containing a final concentration of 50–1000 μM Me-CD) were employed. The order of addition of the component solutions were Me-CD stock, Fe(III) porphyrin stock, and buffer (50 mM citric acid at pH 3.0). All experiments were performed in at least triplicate.

Beer's Law Studies. The absorbance of the tetraaminoporphyrin 3 (0.2–20 μM; 50 mM succinic acid, pH 6.0, 25 °C) was monitored at 406 nm. An analogous series of experiments were performed in the presence of an 800-fold excess of Me-CD.

Solubility Studies. A 20-fold molar excess of Me-CD (266 mg) was added to a solution containing the tetraaminoporphyrin 3 (9.6 mg) in 0.16 M KCl/0.35 M HCl (920 μL). The resultant dark green homogeneous solution was then titrated with 80 μL of 6.25 M KOH to afford a final pH of 13.50.

UV–Visible Studies on the Diprotonated Porphyrin 11. A 20-µL aliquot of the tetraaminoporphyrin 3 (50 µM, 0.16 M KCl/0.04 M HCl, pH 2.0) was added to individual buffered solutions (50 mM succinic acid, pH 4.0) containing a 25–1000-fold molar excess of Me-CD in a final volume of 1.0 mL. The cyclodextrin stock solutions (50 mM succinic acid, pH 4.0) employed were either 1.0 mM (25–300-fold molar excess relative to 3) or 10.0 mM (500–1000-fold molar excess relative to 3) in Me-CD.

Results

The aromatic aldehyde 1 was prepared by the alkylation of 4-hydroxybenzaldehyde with *N*-(3-bromopropyl)phthalimide in DMF/potassium carbonate (84.7% yield). The subsequent reaction of 1 with pyrrole under the standard protocol of Lindsey furnished the 5,10,15,20-tetraphthalimidoporphyrin 2.[5] Treatment
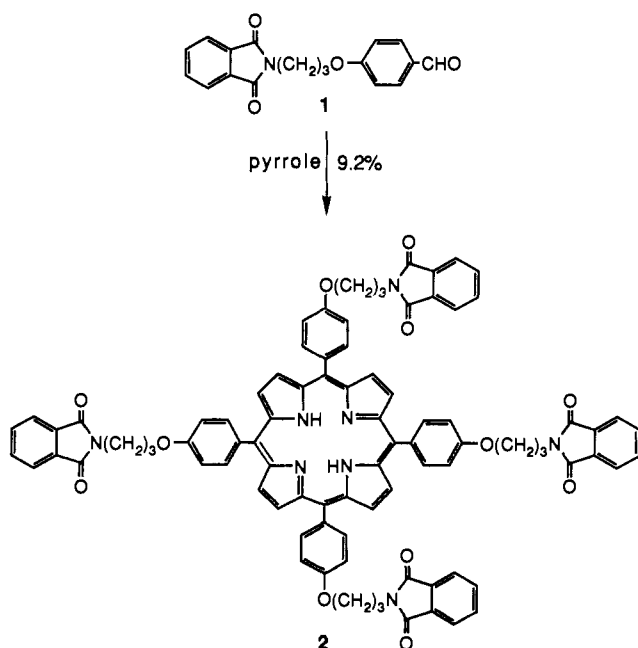
of 2 with hydrazine in THF at reflux for 5 h gave the corresponding tetraaminoporphyrin 3 (85.0%). Iron was inserted at the tetraphthalimido stage (4, 96.0%).[6] The phthalimide protecting groups were then cleaved (67.4%) to furnish the metalloporphyrin 5.
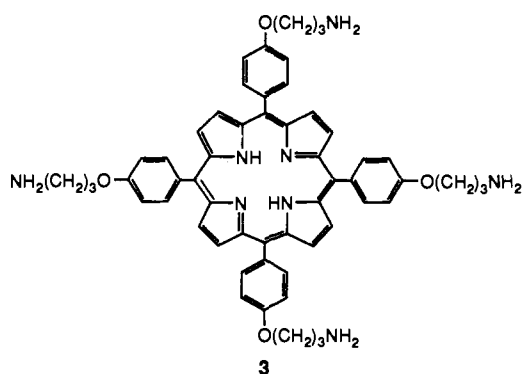
The diarylporphyrin 6 was obtained employing a previously reported protocol.[9] Subsequent modification of this porphyrin, in a fashion analogous to that described above for the tetraarylporphyrins, provided the desired diaminoporphyrin 7 and its metalated complex 8.[10]
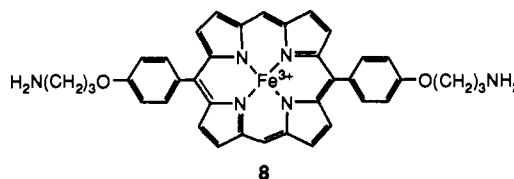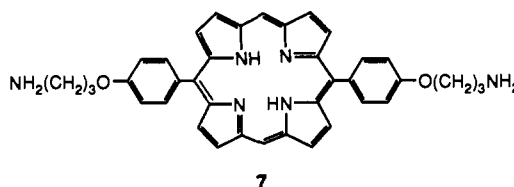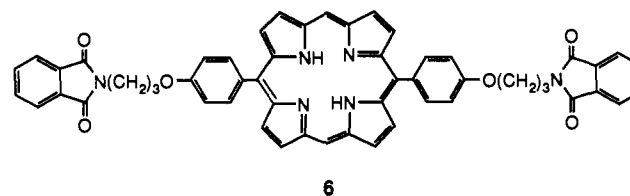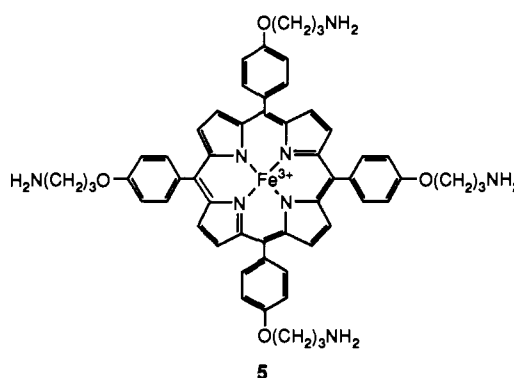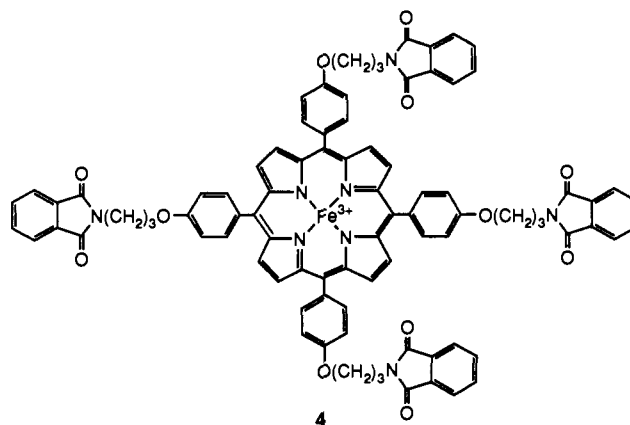

The encapsulation of porphyrin 3 by heptakis(2,6-di-*O*-methyl)-β-cyclodextrin ("Me-CD") 9 was characterized by one- and two-dimensional nuclear magnetic resonance spectroscopies. In the presence of an equimolar ratio of porphyrin, the H-3 and H-5 protons of the cyclodextrin exhibited shifts of 0.12 (upfield) and 0.04 ppm (downfield), respectively. Alterations in the chemical shift for these protons are often observed in the formation of cyclodextrin-based inclusion complexes.[11] The results of two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) studies on this system also reveal that the porphyrin has been incorporated within the interior of the cyclodextrin cavity

(8) Conners, K. A. *Binding Constants. The Measurement of Molecular Complex Stability*; John Wiley & Sons: New York, 1987; p 161–168.
(9) Manka, J. S.; Lawrence, D. S. *Tetrahedron Lett.* **1989**, *30*, 6989.
(10) The synthesis and characterization of 6 and 7 will be described in a future publication. Manka, J. S.; Lawrence, D. S., manuscript in preparation.

(11) For example, see: Wood, D. J.; Hruska, F. E.; Saenger, W. *J. Am. Chem. Soc.* **1977**, *99*, 1735.
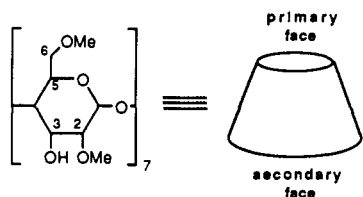
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(unbuffered D_2O pD 4.0). The proton–proton through-space interactions between the cyclodextrin and porphyrin are as follows: H^3 (Me-CD) → β-pyrrole (porphyrin); H^3 (Me-CD) → phenyl H meta to ether (porphyrin); H^3 (Me-CD) → phenyl H ortho to ether (porphyrin); H^5 (Me-CD) → phenyl hydrogen ortho to ether (porphyrin); O^6-CH_3 (Me-CD) → middle (CH_2) of ether side chain (porphyrin). These results indicate that the Me-CD component has encapsulated the porphyrin subunit via the secondary face first (i.e., as depicted in structure 10).
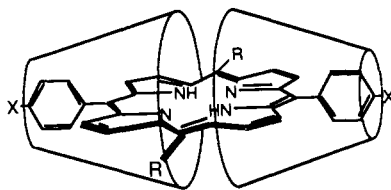

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The interaction of porphyrins 3 and 7 and metalloporphyrins 5 and 8 with Me-CD can be followed by an increase in the absorption of the porphyrin's Soret band as a function of increasing Me-CD concentration. We have previously isolated and characterized a supramolecular complex containing 1 equiv of the tetraaminoporphyrin and 2 equiv of Me-CD.[12] Consequently, in the studies reported herein, we have assumed that encapsulation proceeds via a two-step binding process (Scheme I). The binding constants associated with the 1:1 and 2:1 inclusion complexes of Me-CD and tetraaminoporphyrin 3 are K_{11} = 7.7 ± 0.7 × 10^4 and K_{12} = 5.9 ± 1.1 × 10^4 M^{-1}, respectively (succinic acid buffer, pH 5.0, maintained at 60 °C). The association constants were obtained employing the mathematical treatment previously described by Connors.[7] The binding constants associated with the 1:1 and 2:1 inclusion complexes of Me-CD and Fe(III) tetraaminoporphyrin 5 are K_{11} = 3.5 ± 0.3 × 10^4 and K_{12} = 9.0 ± 3.7 × 10^2 M^{-1}, respectively [(citric acid buffer, pH 3.0, 25 °C (nonthermostatted)]. In this case, the results obtained for Me-CD concentrations at ratios of Me–CD/Fe(III) porphyrin of 30:1 and less were not employed to calculate the binding constants. These data exhibited a curved upward deviation versus that obtained for higher concentrations of Me-CD in the plot of eq 4. This curved deviation is a direct consequence of larger than expected absorbance changes in the Soret band of the Fe(III) porphyrin at relatively low concentrations of variable [Me-CD]. This is most likely a consequence of Me-CD-promoted aggregate breakup in combination with encapsulation. A Beer's law study of 5 at pH 3.0 (citric acid buffer) revealed a slight departure from the expected linear curve (data not shown), which is consistent with the presence of at least some porphyrin aggregate. Finally, association constants for the corresponding diarylporphyrin and its metalated counterpart could not be obtained. This is possibly a consequence of heavy aggregation of these species in the absence of excess Me-CD, since the diarylporphyrins are less sterically hindered than their tetraaryl counterparts.

A Beer's law titration of the 5,10,15,20-tetraaminoporphyrin 3 at pH 6.0 (succinic acid buffer, 25 °C) in the presence and absence of Me-CD is depicted in Figure 1. In the absence of Me-CD, a marked deviation from Beer's law was observed (break point at ~4 μM), indicating that the porphyrin is aggregated. The absorbance measurements were performed at 406 nm, the wavelength at which the aggregated porphyrin absorbs most

(12) Manka, J. S.; Lawrence, D. S. *J. Am. Chem. Soc.* 1990, *112*, 2440.

Scheme I
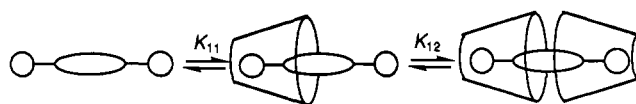
strongly. Consequently, the absorbance of the porphyrin in the absence of Me-CD deviates in an upward fashion from that which is expected for Beer's law behavior. In contrast, in the presence of an 800-fold excess of Me-CD, the photophysical properties of the porphyrin adhered closely to Beer's law. The corresponding Fe(III)-tetrasubstituted porphyrin 5 exhibited behavior which is in sharp contrast to that of 3 in the presence of Me-CD. At pH 4.0 and above, the Fe(III) porphyrin aggregates via the formation of a μ-oxo Fe(III)–O–Fe(III) bridge (UV–vis, 574 and 622 nm; IR[13] 847 and 872 cm^{-1}). Interestingly, excess Me-CD failed to preclude this transformation (data not shown).

All the porphyrins and metalloporphyrins investigated in this study proved to be completely insoluble under basic conditions (pH 13.5). In contrast, in the presence of a 20-fold molar excess of Me-CD, these species remained soluble to a concentration of greater than 10.0 mM.

At pH 4.0 (succinic acid buffer), in the absence of Me-CD, the tetraaminoporphyrin exists primarily as the diprotonated species 11. In this state we did not expect the porphyrin to interact
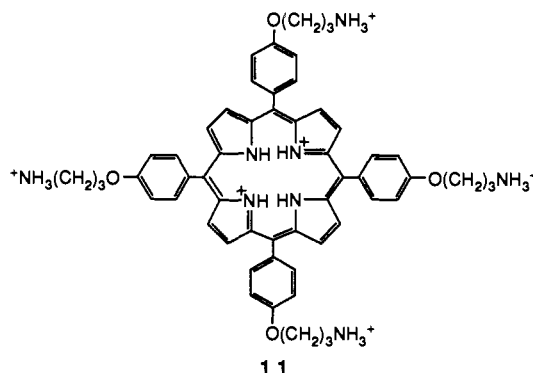
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in a facile fashion with Me-CD, since the latter contains an inner hydrophobic core. However, as is apparent from Figure 2, encapsulation does proceed with concomitant deprotonation of 11. ^{1}H NMR studies, performed under analogous conditions, confirmed that the porphyrin had been encapsulated by the cyclodextrin. The downfield (0.20 ppm) chemical shift of the β-pyrrole protons in 11, induced by the addition of Me-CD, corroborated the notion that the cyclodextrin promotes the conversion of the diprotonated porphyrin 11 to its free base counterpart 3.

Discussion

Both the tetraaminoporphyrin 3 and its diamino counterpart 7 contain opposing aryl substituents of the proper size and shape to bind within the hydrophobic cavity of heptakis(2,6-di-O-methyl)-β-cyclodextrin ("Me-CD") 9 in the fashion depicted in 10.[14] Indeed, we have previously isolated the 2:1 inclusion complex between Me-CD and the tetraarylporphyrin 3.[12,15] As a conse-

(13) Cohen, I. A. *J. Am. Chem. Soc.* 1969, *91*, 1980.

(14) Several reports of porphyrins that are covalently linked to cyclodextrins have appeared: (a) Kobayashi, N.; Akiba, U.; Takatori, K.; Ueno, A.; Osa, T. *Heterocycles* 1982, *19*, 2011. (b) Wife, R. L.; Creed, D. E.; Volger, H. C. In *Proceedings of the First International Symposium on Cyclodextrins*; Szejtli, J., Ed.; Reidel Publishing Co.; Dordrecht, 1981; p 289. (c) Gonzalez, M. C.; Weedon, A. C. *Can. J. Chem.* 1984, *63*, 602. (d) Kato, T.; Nakamura, Y. *Heterocycles* 1988, *27*, 973. (e) Kuroda, Y.; Hiroshige, T.; Takashi, S.; Shiroiwa, Y.; Tanaka, H.; Ogoshi, H. *J. Am. Chem. Soc.* 1989, *111*, 1912.

(15) Cyclodextrins have been shown to form 2:1 complexes with a variety of guest molecules: (a) Kondo, H.; Nakatani, H.; Hiromi, K. *J. Biochem.* 1976, *79*, 393. (b) Vogtle, F.; Muller, W. M. *Angew. Chem., Int. Ed. Engl.* 1979, *18*, 623. (c) Szejtli, J.; Bolla-Pusztai, E.; Szabo, P.; Ferenczy, T. *Pharmazie* 1980, *35*, 779. (d) Harada, A.; Takahashi, S. *J. Chem. Soc., Chem. Commun.* 1986, 1229. (e) Herkstroeter, W. G.; Martic, P. A.; Evans, T. R.; Farid, S. *J. Am. Chem. Soc.* 1986, *108*, 3275. (f) Jobe, D. J.; Verrall, R. E.; Palepu, R.; Reinsborough, V. C. *J. Phys. Chem.* 1988, *92*, 3582. (g) Catena, G.; Bright, F. *Anal. Chem.* 1989, *61*, 905.

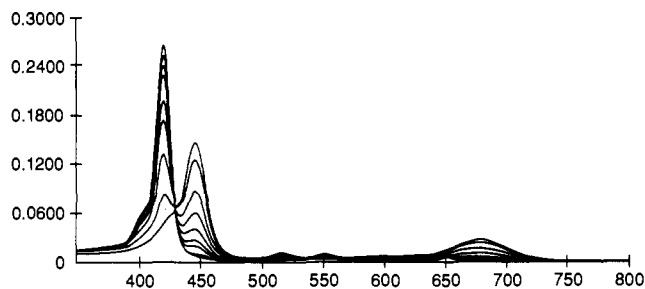2668 *J. Am. Chem. Soc., Vol. 114, No. 7, 1992*

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Figure 2. UV–visible spectra of porphyrin 3 at pH 4.0 (succinic acid buffer) as a function of Me-CD concentration.

quence of this prior work (in conjunction with the related studies of Connors and his co-workers[16]), we reasoned that the porphyrin guest is most likely encapsulated by the Me-CD hosts in the stepwise fashion portrayed in Scheme I. Our initial attempts to obtain the binding constants depicted in this scheme were frustrated by the highly capricious photophysical behavior of both porphyrins 3 and 7 in the presence of varying concentrations of cyclodextrin. We suspect that this may be due, in part, to the presence of porphyrin aggregates in the absence of excess cyclodextrin. We eventually found that a succinic acid buffer (pH 5.0), maintained at 60 °C, provided favorable conditions for obtaining the following association constants: K_{11} of 7.7 ± 0.7 × 10^4 and K_{12} of 5.9 ± 1.1 × 10^4 M^{-1} for the tetraaminoporphyrin 3. These values are substantially larger (2–3 orders of magnitude) than the formation constants generally observed for the inclusion complexes between simple organic guest molecules and underivatized cyclodextrins.[17] Since Me-CD does not appear, in general, to bind organic compounds any more strongly than its unmethylated counterpart,[17] it seems likely that the unusually large association constants obtained in this case are a consequence of interactions which are quite specific to this particular system. Molecular models suggest that the porphyrin can penetrate deeply into the cyclodextrin cavity, thereby providing a favorable hydrophobic environment for the considerably lipophilic porphyrin periphery. The binding constants ($K_{11} = 3.5 ± 0.3 × 10^4$ and $K_{12} = 9.0 ± 3.7 × 10^2$ M^{-1}) for the Fe(III) porphyrin 5 were obtained at room temperature (~25 °C) and pH 3.0. The relatively low pH was employed to preclude μ-oxo dimer formation. Due to the different conditions under which the experiments with the free base porphyrin 3 and its Fe(III)-containing counterpart 5 were performed, the resultant binding constants cannot be compared in the strictest sense. However, in general terms, we note that the K_{11} value for 3 is approximately twice that obtained for 5. The former was acquired at 60 °C and would have likely been approximately 5-fold larger[17] had we been able to perform the experiments on 3 at room temperature. In short, our best estimate is that, under comparable conditions, the K_{11} value associated with the 1:1 Me-CD/porphyrin complex is probably about an order of magnitude larger than the K_{11} for the 1:1 Me-CD/Fe(III) porphyrin complex. Undoubtedly, the positively charged metal atom in 5 renders the porphyrin less lipophilic than its free base counterpart 3. Consequently, it is not too surprising that the Me-CD binds slightly less well to 5 than to 3.

Connors and his colleagues have studied the interaction of α-cyclodextrin with a variety of symmetrically disubstituted 4,4′-biphenyls.[16] Both 1:1 and 2:1 host/guest complexes are generated in this instance as well. An interaction parameter ($a = 4K_{12}/K_{11}$) has been defined for such systems, which is a measure of "the interaction between the two (binding) sites in 1:2 complex formation".[16] For example, if the binding sites are acting independently of each other, then $a = 1$. In the case of Me-CD and the porphyrin 3, we observe a slight positive cooperativity ($a = 3.0$) in the stepwise binding process, indicating that Me-CD ex-

hibits a somewhat stronger affinity for the 1:1 Me-CD–porphyrin complex than for the free porphyrin alone. Electronic factors or host–host interactions in the 2:1 complex could account for this behavior. However, in thermodynamic terms, the observed positive cooperativity is not particularly strong (if $a = 1$, then K_{12} would be 3-fold less than its experimentally obtained value, which translates into a 0.75 kcal/mol advantage for the formation of the 2:1 complex versus the 1:1 complex). In contrast, a definite negative cooperativity ($a = 0.10$) is apparent in the stepwise binding process with the Fe(III) porphyrin 5. In short, the formation of the 2:1 Me-CD/Fe(III)-porphyrin complex ($K_{12} = 900$ M^{-1}) is not as strongly favored as the formation of the corresponding 1:1 complex ($K_{11} = 34\,000$ M^{-1}). In the former case, this may simply reflect the incorporation of a positively charged metalloporphyrin into a hydrophobic microenvironment, which is formed when *two* cyclodextrins associate with the heme. In the 1:1 complex, the metal ion itself remains well exposed to the aqueous environment. These results (consistent with molecular models) imply that two cyclodextrins are required to create the requisite hydrophobic environment about the porphyrin nucleus. Therefore, one might expect that a neutral porphyrin would form the 2:1 complex more readily than the corresponding charged metalloporphyrin. Indeed, the free base porphyrin 3, which lacks the ferric ion, exhibits a K_{12} for the formation of the 2:1 species that is 60-fold larger (more likely 300-fold larger had both K_{12} values been obtained at room temperature) than that for the Fe(III) porphyrin 5.

Analogous studies to those described above were attempted on the 5,15-diarylporphyrin 7 and its Fe(III)-containing analogue 8. Although we investigated a wide variety of conditions, we were unable to obtain reproducible association constants for the assembly of 10 in these two cases. These difficulties may be a consequence of porphyrin aggregation in the absence of excess cyclodextrin, which would not be surprising since the diarylporphyrins are much less sterically hindered than their tetraaryl counterparts.

The structure of the porphyrin–cyclodextrin complex was established by NMR spectroscopy. As expected, the resonances of the protons at C-3 (0.12 ppm upfield) and C-5 (0.04 ppm downfield) of the Me-CD undergo a chemical shift upon introduction of porphyrin 3. Two-dimensional NOESY spectroscopy confirmed that the porphyrin is complexed by Me-CD. The β-pyrrolenic protons of the porphyrin experience a through-space interaction with the protons positioned at C-3 (but not C-5) on the cyclodextrin. In addition, these NMR experiments also indicate that the central methylenes of the porphyrin's aminopropyl side chains are situated near the methyl groups on the primary face of the cyclodextrin component. Consequently, it is evident that the porphyrin moiety entered the cyclodextrin cavity via the secondary face of the saccharide.

We now report on the ability of the cyclodextrin sheath to serve, at least in part, as a substitute for the protein component of heme-containing proteins.

The cyclodextrin sheath as a sequestering agent, precluding undesirable porphyrin–porphyrin interactions:[18] The cytochrome P-450s, peroxidases, and catalases all contain a metalloporphyrin moiety embedded within a protective pocket. In the resting state of the enzyme, the structural integrity of the metalloporphyrin is preserved, in part, due to the steric constraints imposed by the protein component which prevents porphyrin–porphyrin interaction.[19] Indeed, many simple unhindered water-soluble porphyrins rapidly aggregate in the absence of steric constraints upon introduction into an aqueous environment.[20,21] Aggregation

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(17) Gelb, R. I.; Schwartz, L. M. *J. Incl. Phenom. Mol. Recog. Chem.* **1989**, *7*, 537.

(18) While this paper was under revision a report appeared which described the β-cyclodextrin-promoted decomposition of an Fe(III) porphyrin μ-oxo dimer. See: Mosseri, S.; Mialocq, J. C.; Perly, B.; Hambright, P. *J. Phys. Chem.* **1991**, *95*, 4659.

(19) The protein component of hemoglobin also acts as a steric barrier. Unhindered Fe(II) porphyrins are able to bind molecular oxygen, but they do so only transiently, since they rapidly dimerize to form μ-oxo linked hemes, species which can no longer bind oxygen. Jones, R. D.; Summerville, D. A.; Basolo, F. *Chem. Rev.* **1979**, *79*, 139.

(particularly for Fe porphyrins) interferes with the ability of the monomeric species to carry out such tasks as oxygen transport and activation. Our studies were performed on the free base porphyrin **3**. In the absence of cyclodextrin, the tetraaminoporphyrin failed to obey Beer's law (Figure 1), indicating that the porphyrin is aggregated (pH 6.0). In contrast, in the presence of an 800-fold excess of Me-CD, the photophysical behavior for the tetraaminoporphyrin is in accordance with Beer's law (Figure 1). This is consistent with the notion that the cyclodextrin moiety serves as a sequestering agent, precluding porphyrin–porphyrin interactions.[22]

The cyclodextrin sheath as a carrier, rendering the porphyrin water-soluble under conditions in which the isolated species is insoluble: Some heme-dependent proteins (e.g., catalase) are water-soluble, functioning as carriers for otherwise water-insoluble hemes. Interestingly, both the Fe(III) porphyrin **5** and its corresponding free base **3** are almost completely water-insoluble under conditions of moderate-to-high pH. In contrast, the Me-CD moieties render both species freely soluble (>10 mM) in aqueous base (pH 13.5).

The cyclodextrin sheath as an environmental determinant, enveloping the porphyrin within a nonpolar microenvironment: The active sites of hemoglobin and the cytochrome P-450s are thought to be relatively hydrophobic. In the former case, such an environment preserves the Fe(II) oxidation state that is essential for the coordination of oxygen and, in the latter case, the nonpolar active site provides a suitable milieu for lipophilic substrates.

The tetraaminoporphyrin **3** exists primarily as species **11** [henceforth referred to as the "protonated porphyrin"; the pK_a of the pyrrole nitrogens in tetrakis(p-methoxy)tetraphenylporphine in aqueous DMF is 2.3[23]] in 50 mM succinic acid at pH 4.0 (Figure 2; two absorption bands in the 500–700-nm region, as opposed to four bands, and the red-shifted λ_{max} of the Soret band, are characteristic of the diprotonated state).[24] On the basis of the results described above with the Fe(III) porphyrin (specifically the small K_{12}), one might not expect the Me-CD to efficaciously bind the doubly protonated porphyrin moiety.[25] However, at pH

4.0, encapsulation does proceed (analogous results were obtained at pH 3.0 in a citric acid buffer). Interestingly, the encapsulation phenomenon is strongly linked to the simultaneous conversion of the protonated porphyrin to its free base counterpart (Figure 2)! The Me-CD, with its hydrophobic interior, may preferentially bind any residual unprotonated neutral porphyrin present in solution under these conditions. The equilibrium concentration ratio of free protonated and free unprotonated porphyrins would then be reestablished, producing a greater total concentration of unprotonated porphyrin (i.e., both cyclodextrin-bound and free forms). Alternatively, the Me-CD may directly encapsulate the protonated porphyrin, followed by the subsequent deprotonation of the latter. In either case, the cyclodextrin-promoted formation of neutral porphyrin under acidic conditions is consistent with the results of previously reported studies that have established the hydrophobic nature of the cyclodextrin interior.[26]

NMR studies offer some further insight into the encapsulation process under acidic conditions (unbuffered D_2O, pD 4.0). The chemical shift of the β-pyrrolenic protons is mildly sensitive to the protonation state of the porphyrin moiety. For example, previously reported experiments on tetraphenylporphyrin have revealed that these protons experience an upfield shift of 0.08 ppm when the porphyrin is protonated.[27] The addition of Me-CD to the protonated porphyrin **11** results in the expected downfield shift (0.20 ppm) of the β-pyrrolenic protons, suggesting that the porphyrin has been converted to the unprotonated state (confirmed by UV–visible studies). Furthermore, the cyclodextrin is responsible for this transformation since, in the presence of 1 equiv of Me-CD, the β-pyrrolenic protons are coupled through-space to the protons at C-3 on the cyclodextrin component. These experiments demonstrate that encapsulation has a profound effect upon the acid/base properties of the porphyrin moiety. Perhaps even more significantly, upon addition of an excess of Me-CD, the resultant UV–visible spectrum of the porphyrin is identical to those spectra obtained for the unprotonated porphyrin in such nonpolar organic solvents as dioxane and benzene (data not shown). These results suggest that the cyclodextrin sheath envelops the porphyrin within a nonpolar environment.

It is important to note that, in some of the experiments described above, a large excess of Me-CD was required to ensure that the porphyrin nucleus was completely encapsulated by two cyclodextrin moieties. We have recently shown that the inclusion complex **10** can be sequestered, thereby circumventing the requirement for excess Me-CD.[12] We are currently investigating the biologically relevant activity of related species.

In summary, we have characterized a simple three-component molecular assemblage which possesses some of the properties that are commonly associated with heme-containing proteins. The cyclodextrin sheath not only retards porphyrin aggregation but, in addition, serves as a water-soluble carrier while maintaining a nonpolar inner microenvironment. In this regard, the cyclodextrin sheath appears to be functioning as the molecular equivalent of a micelle.[28]

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(21) White, W. I. *The Porphyrins*; Dolphin, D., Ed.; Academic Press: New York, Vol. V, Chapter 7, p 303.

(22) Interestingly, we failed to obtain analogous results with the Fe(III) tetraaminoporphyrin **5**. Simple unhindered water-soluble Fe(III) porphyrins rapidly dimerize via the formation of a μ-oxo bridge at pH $\geq$ 6.0.[21] At pH 3.0, compound **5** exhibited monomeric behavior (characteristic λ_{max} values, 532 and 690 nm). However, we have found that even at pH 4.0, the Fe(III) porphyrin **5** exists, to at least some extent, in the μ-oxo-dimer form (characteristic λ_{max} values, 574 and 622 nm; characteristic infrared bands, 847 and 872 cm^{-1}). Above pH 6.0 the heme is completely dimerized. Dimerization occurred even in the presence of excess Me-CD. This stands in stark contrast to our results with the free base porphyrin and the results described in ref 18. Since it is highly unlikely that dimerization occurs when the Me-CD moieties are bound to the Fe(III)-porphyrin (based on our studies with the metal-free porphyrin **3**), we suspect that the 2:1 complex must first dissociate to allow dimerization to proceed. This indicates that the association constant for dimerized product exceeds that for the 2:1 complex. The obvious way to preclude μ-oxo dimer formation would be to secure the cyclodextrins permanently to the porphyrin core.[12]

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(24) Smith, K. M. *Porphyrins and Metalloporphyrins*; Smith, K. M., Ed.; Elsevier: New York, 1975; p 24.

(25) Alternatively, the behavior shown in Figure 2 may be a consequence of the conformational state of the diprotonated porphyrin, which is severely puckered (Fleischer, E. B. *Acc. Chem. Res.* **1970**, *3*, 105). In short, it may be the nonplanarity of the porphyrin ring system (the β-pyrrole carbons can be as much as 1 Å out of the plane containing the four pyrrole nitrogen atoms) and not the double positive charge per se, which interferes with the ability of the diprotonated species to interact with Me-CD in a favorable fashion. While we cannot completely rule out this possibility, we note that the Fe(III) porphyrin **5**, which is both planar and charged, exhibits a much smaller K_{12} than the neutral free base porphyrin. This indicates that the presence of a positive charge itself, contained within the porphyrin core, can preclude a strong interaction with the cyclodextrin moiety. In addition, the diameter of the opening on the secondary face of the cyclodextrin is approximately 10 Å, more than large enough to accommodate the puckered porphyrin molecule.

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