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Heme-Protein Active Site Models via Self-Assembly in Water

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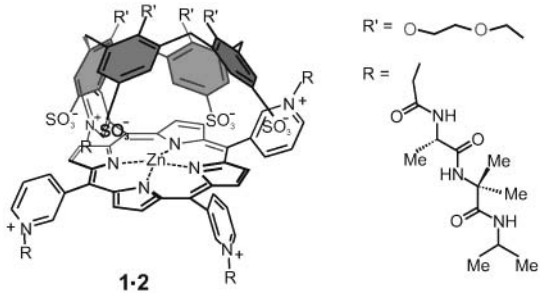
ABSTRACT



Water-soluble models of heme-protein active sites are obtained via the self-assembly of cationic porphyrins 1 and tetrasulfonato calix[4]arene 2 ($K_{1\cdot2} = 10^5$ M^{-1}). Selective binding of ligands either outside or inside the cavity of assemblies 1·2 via coordination to the zinc center has been observed. Small ligands such as 4-methylpyridine and 1-methylimidazole are encapsulated, while the bulkier caffeine is bound outside. Assemblies Co-1·2, in which the Zn porphyrin moiety has been replaced by a Co^{II} porphyrin, can act as O_2 carriers.

Synthetic models of heme-protein active sites have been investigated extensively in order to understand the basic features associated with the functioning of different biological systems.[1−3] From the structural point of view, the active site of a heme-protein is essentially a hydrophobic cavity of appropriate dimensions enclosed between the protein matrix and the metal porphyrin. There are two different approaches for the mimicry of heme-protein active sites, either via covalent[1,2] or noncovalent[4,5] synthesis. However, most of the synthetic model systems suffer from the limitation of poor water-solubility. The main advantage of the noncovalent approach is that, in addition to the synthetic simplicity, it allows a more accurate mimicry of the natural heme-proteins.[6] In this publication, we present the first example of simple analogues of heme-protein active sites obtained by self-assembly of cage-like complexes via ionic interaction in water.[7]

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(1) Collman, J. P.; Fu, L. *Acc. Chem. Res.* **1999**, *32*, 455−463. Tani, F.; Matsu-ura, M.; Nakayama, S.; Ichimura, M.; Nakamura, N.; Naruta, Y. *J. Am. Chem. Soc.* **2001**, *123*, 1133−1142. Smeets, S.; Asokan, F.; Motmans, F.; Dehaen, W. *J. Org. Chem.* **2000**, *65*, 5882−5885. Schwenninger, R.; Ramondenc, Y.; Wurst, K.; Schlögl, J.; Kräutler, B. *Chem. Eur. J.* **2000**, *6*, 1214−1223.

(2) Hecht, S.; Vladimirov, N.; Fréchet, J. M. J. *J. Am. Chem. Soc.* **2001**, *123*, 18−25. Jiang, D.-L.; Aida, T. *J. Am. Chem. Soc.* **1998**, *120*, 10895−10901. Weyermann, P.; Gisselbrecht, J.-P.; Boudon, C.; Diederich, F.; Gross, M. *Angew. Chem., Int. Ed.* **1999**, *38*, 3215−3219. Feiters, M. C.; Rowan, A. E.; Nolte, R. J. M. *Chem. Soc. Rev.* **2000**, *29*, 375−384 and references therein.

(3) Lombardi, A.; Nastri, F.; Pavone, V. *Chem. Rev.* **2001**, *101*, 3165−3189.

(4) Dick, D. L.; Rao, T. V. S.; Sukumaran, D.; Lawrence, D. S. *J. Am. Chem. Soc.* **1992**, *114*, 2664−2669. Michels, J. J.; Fiammengo, R.; Timmerman, P.; Huskens, J.; Reinhoudt, D. N. *J. Incl. Phenom.* **2001**, *41*, 163−172.

(5) Norsten, T. B.; Chichak, K.; Branda, N. R. *Tetrahedron* **2002**, *58*, 639−651.

(6) Huffman, D. L.; Suslick, K. S. *Inorg. Chem.* **2000**, *39*, 5418−5419.

(7) Fiammengo, R.; Crego-Calama, M.; Reinhoudt, D. N. *Curr. Opin. Chem. Biol.* **2001**, *5*, 660−673. Fiammengo, R.; Timmerman, P.; de Jong, F.; Reinhoudt, D. N. *Chem. Commun.* **2000**, 2313−2314.

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We have previously shown the formation of strong 1:1 complexes between tetracationic Zn-porphyrinates and **2** in polar organic solvents.[8] Unfortunately, the solubility of the assemblies in pure water was too low. Now we have changed the design which allows the self-assembly process to occur in pure water. The short peptides attached to the pyridyl nitrogens of porphyrins **1** (Figure 1) are responsible for the relatively large water solubility of assemblies **1·2** (concentrations up to 2−5 mM). These ensembles exhibited association constants $K_{\mathbf{1}\cdot\mathbf{2}}$ in aqueous carbonate buffer solution as high as 10^5 M^{-1}.

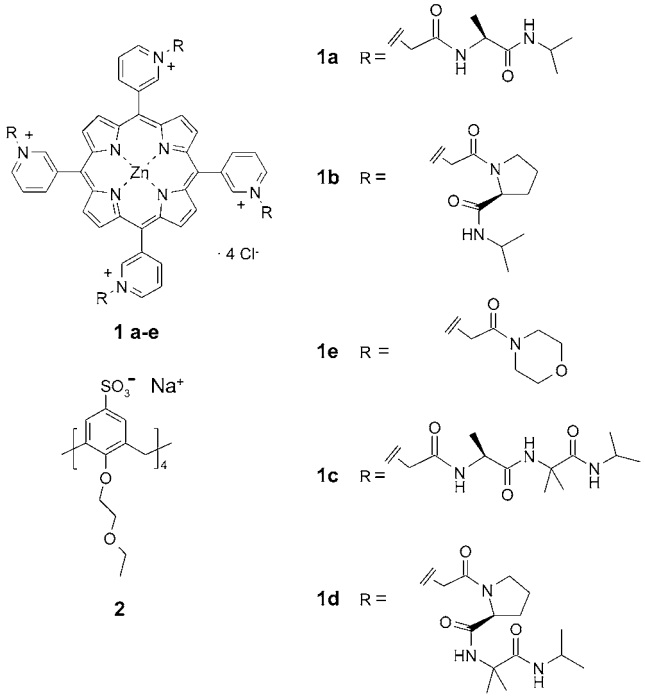

Figure 1.

In previous noncovalent synthetic models for heme-protein[4] the binding properties of the metal porphyrin moiety were not reported. We have previously demonstrated that self-assembled cage-like porphyrin−calix[4]arenes complexes form ternary complexes upon addition of nitrogenous ligands.[8] Binding of the added ligands to the Zn−porphyrin moiety in polar solvents consistently occurred at the solvent-exposed porphyrin face. In contrast, the data reported here show that, using water as solvent, coordination of nitrogenous bases to the Zn center affords ternary complexes with topologies directed by the sterical requirements of the base. Small bases such as 4-methyl pyridine are complexed preferentially inside the hydrophobic cavity of the assemblies, while larger molecules such as caffeine are bound outside the cavity at the solvent-exposed porphyrin face. Moreover, Co^{II} porphyrins can also be self-assembled with **2**, thus opening the way toward *functional* models of heme-protein active sites.

Assembly Formation: ITC Studies. Self-assembly of Zn porphyrins **1a**−**e**[9] with calix[4]arene tetrasulfonate **2**[8] was studied in aqueous solution using isothermal titration microcalorimetry (ITC). The association constants (see Table 1) are quite insensitive to the nature of the N-substituents (the largest difference is less than a factor of 2).

Table 1. Association Constants ($K_{\mathbf{1}\cdot\mathbf{2}}$) for **1a**−**e** (0.090−0.105 M) and **2** at 25 °C

	log $K_{\mathbf{1}\cdot\mathbf{2}}$	$\Delta H°$ (kJ/mol^{-1})	$\Delta S°$ (J mol^{-1} K^{-1})
1a·2[a]	4.90 ± 0.02	−23.9 ± 0.3	−14 ± 1
1a·2[b]	4.26 ± 0.04	−33 ± 3	−29 ± 12
1b·2[a]	4.82 ± 0.02	−32.5 ± 0.3	−17 ± 1
1b·2[b]	4.40 ± 0.02	−36 ± 1	−38 ± 5
1c·2[a]	4.68 ± 0.02	−25.8 ± 0.4	−3 ± 2
1c·2[b]	4.11 ± 0.03	−43 ± 2	−64 ± 8
1d·2[a]	4.90 ± 0.02	−28.9 ± 0.3	+3 ± 1
1d·2[b]	4.23 ± 0.02	−40 ± 1	−54 ± 5
1e·2[a]	4.69 ± 0.01[c]	−25.5 ± 0.3	+4.6 ± 1
	5.62 ± 0.05[d]	−20.8 ± 0.3	+37.7 ± 2
	5.43 ± 0.08[e]	+13.2 ± 0.4	+148 ± 3
Co-**1b·2**[b]	3.80 ± 0.04	−42 ± 4	−71 ± 15

[a] Carbonate buffer, pH = 9.6, I = 0.010 M. [b] Carbonate buffer, pH = 10, I = 0.045 M. [c] Carbonate buffer pH = 9.7, I = 0.020 M. [d] No added salts. [e] MeOH/water (x_{water} = 0.45), 1 × 10^{-2} M Bu_4NClO_4.[8]

Self-assembly of complexes **1a·2** through **1d·2** in carbonate buffered solutions (I = 0.010 M) is characterized by a negative enthalpy and small entropic contributions. However, at higher buffer concentrations (I = 0.045 M), the formation of these complexes is accompanied by larger negative enthalpy and entropy changes. The changes tend to compensate for each other, and the net result is a moderate decrease of the association constants (2−5 times, $\Delta\log K_{\mathbf{1}\cdot\mathbf{2}} \sim 0.4-0.7$). Measurements on the less soluble assembly **1e·2** also confirm this correlation. In the absence of buffer, formation of **1e·2** is even less enthalpically favored but much more entropically favored. The overall effect is an association strength approximately 1 order of magnitude higher ($\log K_{\mathbf{1}\cdot\mathbf{2}}$ = 5.62 vs 4.69 in the presence of carbonate buffer, I = 0.020 M). These observations suggest that assembly formation in aqueous solution is primarily driven by multiple ionic interactions between the two building blocks.

In polar solvents and aqueous solutions the contribution of desolvation is as relevant as (and sometimes even more important than) the contribution associated with complexation. Therefore, the balance between these two contributions is the reason for the rather large differences observed in the measured thermodynamic parameters when the same assembly process takes place in different media. Here, the data

(8) Fiammengo, R.; Timmerman, P.; Huskens, J.; Versluis, K.; Heck, A. J. R.; Reinhoudt, D. N. *Tetrahedron* **2002**, *58*, 757−764.

(9) For experimental details, see Supporting Information. Also see: Fiammengo, R.; Crego-Calama, M.; Timmerman, P.; Reinhoudt, D. N. *Chem. Eur. J.* **2003**, 9, 784−792. Solubility of assemblies **1(d,e)·2** are ≤1 mM due to the higher hydrophobicity of the porphyrin moieties.

suggest that at lower salt concentrations (resulting in a more ordered solvent shell around the ionic groups), the importance of desolvation in driving the assembly process becomes greater. For instance, desolvation dominates the self-assembly of **1e·2** in MeOH/water ($x_{water} = 0.45$) with 0.01 M of Bu_4NClO_4 resulting in a positive $\Delta H° = +13.2$ kJ/mol and positive $\Delta S° = +148$ mol^{-1} K^{-1}).[8]

UV−Vis Studies. Addition of a solution of calix[4]arene **2** to **1e** in aqueous carbonate buffer ($I = 7.2$ mM)[10] produces spectral variations in the porphyrin Soret band region (400−480 nm) similar to what was previously observed in methanol.[8] The spectral variations (bathochromic shift $\Delta\lambda_{max} = 2-3$ nm, $\Delta\epsilon$ at $\lambda_{max} = 10-15\%$) could be fitted to a 1:1 model.

Formation of assembly **1e·2** was observed in basic, neutral, and acidic solutions. The presence of various electrolytes had no influence on the association constant $K_{\mathbf{1\cdot2}}$ (Table 2).

Table 2. Association Constants ($K_{\mathbf{1e\cdot2}}$ in M^{-1}) for the Formation of **1e·2** in Water at 25 °C, (UV−Vis)

medium	log $K_{\mathbf{1\cdot2}}$
$NaNO_3$, $I \sim 10$ mM, pH = 8.0	5.28 ± 0.03
$NaNO_3$, $I \sim 10$ mM, pH = 4.2[a]	5.24 ± 0.02
$NaNO_3$, $I \sim 10$ mM, pH = 9.5[b]	5.24 ± 0.02
phosphate buffer, $I = 8.0$ mM, pH = 7.0	5.23 ± 0.02
carbonate buffer, $I = 7.2$ mM, pH = 9.4	5.38 ± 0.01
CH_3OH[8]	6.86 ± 0.06

[a] pH adjusted with 0.1 M HCl. [b] pH adjusted with 0.1 M NaOH.

These data compare well with those obtained via ITC considering the slightly different conditions (log $K_{\mathbf{1\cdot2}} = 5.62$ in the absence of added salts and 4.69 in carbonate buffer $I = 0.020$ M; see Table 1).

Conductometric Studies. Changes in conductivity of the solution were observed during the titration of **1e** with **2** due to the formation of the ion-pair complex. The titration endpoint at 0.85 equiv is in reasonable agreement with the proposed 1:1 stoichiometry (see Figure 1 in Supporting Information).

^{1}H NMR Studies. The addition of 1 equiv of **2** to **1c** in D_2O/CD_3OD (9:1) produces large upfield shifts of the calix[4]arene protons due to the anisotropic shielding from the porphyrin ring. The methylene bridges (H_b; see Figure 2 in Supporting Information) and the CH_2 directly connected to the lower rim oxygens (H_c) are the most upfield shifted ($\Delta\delta = 0.7-0.85$ ppm), due to their closer position to the porphyrin center.[11] Moreover, all the signals are significantly broadened, indicating an exchange equilibrium rate approaching the chemical shift time scale. The porphyrin pyridyl ring signals (H_{d-g}) are also shifted upfield ($\Delta\delta$ 0.15−0.40 ppm) and significantly changed in their appearance, indicating the existence of several slowly interconverting isomers.[8] However, the signals relative to the *i*-Pr groups (Me^{i-Pr} and H^{i-Pr}) move in the opposite direction (downfield). Preliminary studies on ligand binding to tetracationic porphyrins **1c−d** showed that in aqueous solution, the rather hydrophobic dipeptide chains are not freely exposed to the bulk solvent but organized to some extent in the proximity of the porphyrin.[9] Evidence for these interactions is the unusual high-field resonances for the *i*-Pr groups terminating the peptidic chains.[12] The observed downfield shifts are therefore attributed to the displacement of the peptide chains from the porphyrin plane caused by the assembly formation.

Selective Binding of Nitrogenous Ligands.[13] In natural heme-protein, the position occupied by the porphyrin within the peptidic matrix is such that the two different porphyrin faces can be distinguished. At the vicinal site, coordination of an amino acid residue to the metal center takes place, while the more open nature of the distal site allows binding of exogenous ligands. Our self-assembled heme-protein mimics **1·2** present two differentiated porphyrin faces, one enclosing a small cavity in conjuction with calix[4]arene **2** and the other open to the solvent (but susceptible to the influence of the peptidic chains, vide infra). UV−vis measurements were used to study the formation of ternary complexes between porphyrins **1**, calix[4]arene **2**, and a ligand **L**. The experimental data were fitted to a 1:1:1 mathematical model encompassing the simultaneous occurrence of three equilibria in solution (eqs 1−3).[14]

$$\mathbf{1} + \mathbf{2} \leftrightarrows \mathbf{1\cdot2} \qquad K_{\mathbf{1\cdot2}} \tag{1}$$

$$\mathbf{1} + \mathbf{L} \leftrightarrows \mathbf{1\cdot L} \qquad K_{\mathbf{1\cdot L}} \tag{2}$$

$$\mathbf{1} + \mathbf{2} + \mathbf{L} \leftrightarrows \mathbf{1\cdot2\cdot L} \qquad K_{\mathbf{1\cdot2\cdot L}} \tag{3}$$

The coordination strength of the axial ligand **L** to porphyrin **1** ($K_{\mathbf{1\cdot L}}$) was determined in separate experiments. The affinity displayed by the assembly **1·2** for a ligand **L** is given by the ratio $K_{\mathbf{1\cdot2\cdot L}}/K_{\mathbf{1\cdot2}} = K_{rec}$. Accordingly, $K_{rec} = 0.5 \times K_{\mathbf{1\cdot L}}$ if **2** is completely blocking one porphyrin side without any other effect on the metal coordination ability (this conclusion should apply to large guests that cannot be encapsulated). On the other hand, for covalently capped Zn−porphyrins,[15] it has been observed that complexation of guests of appropriate dimension within the host cavity leads to enhanced binding affinity and therefore $K_{rec} \geq K_{\mathbf{1\cdot L}}$ should be expected. However, the magnitude of this effect is

(10) [**1e**] = $2-3 \times 10^{-6}$ M. [**2**] = 8.3×10^{-4} M. Very good linearity was observed in dilution experiments ($8 \times 10^{-5}-1 \times 10^{-7}$ M), confirming nonaggregation of the porphyrin. Hydrophilic anions were chosen because anions such as borates and perchlorates were causing aggregation.

(11) The closer position of protons H_b and H_c to the porphyrin center can be better visualized in the molecular simulation structure (CHARMm 24.0) of assembly **1·2** (see Figure 3 in Supporting Information).

(12) In contrast, **1a**,**b** bearing single amino acid-based (short) chains do not show these interactions.

(13) Basic pH was chosen because it is the same media where complexation of nitrogenous bases is also studied in order to neglect the protonation equilibria of the bases. $I \sim 0.008$ M for the titrated porphyrin solution was chosen to equalize the ionic strength of the calix[4]arene solution (8×10^{-4} M) used as a titrant. Constant ionic strength is ensured throughout the titration while keeping at a minimum level the amount of added salts, which decreases $K_{\mathbf{1\cdot2}}$.

(14) Binding of the neutral ligands to **2** has been excluded according to Arena, G.; Contino, A.; Gulino, F. G.; Magrì, A.; Sciotto, D.; Ungaro, R. *Tetrahedron Lett.* **2000**, *41*, 9327−9330.

(15) Weiss, J. *J. Incl. Phenom.* **2001**, *40*, 1−22. Robertson, A.; Shinkai, S. *Coord. Chem. Rev.* **2000**, *205*, 157−199.

determined by the rigidity of the host, the possibility of additional specific interactions between the ligand and the porphyrin superstructure (e.g., H-bonds), and by the solvent.

A small, axially symmetrical base such as 4-methylpyridine (4-MePyr) binds to **1c·2** and **1e·2** with increased affinity (K_{rec} = 58 and 38 M^{-1}, respectively) when compared to **1c** and **1e** ($K_{1\cdot L}$ = 35 and 7 M^{-1}, respectively), suggesting ligand encapsulation ($K_{rec} > K_{1\cdot L}$) (Table 3). However, the rather large difference in $K_{1\cdot L}$ for 4-MePyr displayed by **1c** versus the very similar **1e** suggests that not only metal coordination influences the binding but that additional factors are also playing a role. This difference in affinity is consistent with the previously observed increased binding of small ligands (such as 1-methylimidazole) to porphyrin **1c**, caused by the folding of the long N-substituted chains toward the porphyrin core.[9] Therefore, to ascertain the real increase in affinity obtained upon encapsulation of 4-MePyr by **1c·2**, K_{rec} should instead be compared to $K_{1\cdot L}$ for **1e** in order to eliminate the bias caused by this phenomenon. The result is enhanced binding by a factor of 8 attributable to encapsulation. Similar UV−vis experiments with **1e·2** showed encapsulation of a different guest, 1-MeIm, with K_{rec} four times higher than $K_{1\cdot L}$.

Table 3. Binding Constant for Nitrogenous Bases to **1c·2** and **1e·2** and to **1c**,**e** (Carbonate Buffer, I = 0.008 M, pH = 9.6, T = 25 °C)

assembly	guest	K_{rec} (M^{-1})	porphyrin	$K_{1\cdot L}$ (M^{-1})
1c·2[a]	4-MePyr	$(5.8 \pm 0.5) \times 10^1$	**1c**	$(3.5 \pm 0.1) \times 10^1$
1e·2[a]		$(3.8 \pm 0.3) \times 10^1$	**1e**	$(0.7 \pm 0.1) \times 10^1$
1c·2[b]	caffeine	$(0.80 \pm 0.1) \times 10^3$	**1c**	$(3.26 \pm 0.05) \times 10^3$
1e·2[a]	1-MeIm	$(2.0 \pm 0.3) \times 10^2$	**1e**	$(4.5 \pm 0.2) \times 10^1$

[a] Prepared in situ from **1c** (or **1e**) and 1.2−1.5 equiv of **2**. [b] Prepared in situ from **1c** and 11 equiv of **2** (see Supporting Information).

^{1}H NMR measurements support that complexation is taking place in the cavity (see Figure 4 in Supporting Information). Addition of 1 equiv of 4-MePyr to a 1 mM solution of assembly **1c·2** (from equimolar amounts of **1c** and **2**) in D_2O/CD_3OD 9:1 results in upfield-shifted 4-MePyr proton signals when compared to the spectrum of the guest alone. The signal for proton H_h, ortho to the nitrogen atom, is the most upshifted ($\Delta\delta \sim 0.4$ ppm).[16] This shift, caused by the porphyrin ring current, is also observed in the case of complexation of 4-MePyr to porphyrin **1c** alone. However, small but reproducible upfield shifts ($\Delta\delta$ = 0.05−0.06 ppm) are observed for protons H_i and H_l when binding of 4-MePyr to **1c·2** is compared to binding to **1c**. This observation supports the formation of the inclusion complex with the observed shifts caused by the shielding from the calix[4]-arene moiety. Additionally, proton H_h, (closer to the porphyrin, but farther away from the calix[4]arene) does not show any additional shift when binding to **1c·2** is compared to **1c**.

Moreover, complexation experiments using the bulkier ligand caffeine show no encapsulation (see Figure 5 and Table 1 in Supporting Information). From UV−vis titrations, a K_{rec} = 800 M^{-1} for the self-assembled receptor **1c·2** was calculated. This value is one-fourth the value of $K_{1\cdot L}$ for **1c** (3260 M^{-1}). The decreased affinity confirms that caffeine is too big to fit in the cavity and that binding is taking place only on the open face of the self-assembled receptor. This decrease also suggests larger unfavorable interactions with the long peptidic chains, which are probably arranged predominantly on the open face of the porphyrin because of the ion-pair complex **1c·2** formation (vide supra).[9] (For structural details, see Supporting Information.)

O_2 Transport. It is well-known that Co^{II} porphyrins are able to bind O_2 and have been used as models for natural heme-proteins and as synthetic O_2 carriers.[1] Surprisingly, there are no examples reported of biomimetic O_2 binders and transporters obtained via noncovalent synthesis in water. Thus, the ability of the self-assembled carrier Co-**1b·2**·1-MeIm (2.0×10^{-3}, 6.0×10^{-3}, and 2.0×10^{-2} M, respectively, for the three building blocks in water) to transport O_2 was studied.[17] Preliminary measurements showed a modest but significant facilitated O_2 transport (facilitation factor = 1.15, selectivity coefficient = 2.3−2.4) of the membrane loaded with the carrier with respect to the membrane loaded with only H_2O (facilitation factor = 1.0, selectivity = 2.0, see Figure 6 Supporting Information). Several control experiments showed that the enhancement observed in O_2 transport occurs only if all the components constituting the self-assembled carrier are present. Further investigations of these systems are currently underway in our laboratories.

In conclusion, the formation of self-assembled cage-like complexes in aqueous solution has been achieved. The formation of the assembly is primarily driven by ionic interaction. The assembly process does not involve the metal center, and formation of ternary complexes upon addition of suitable ligands has been achieved. The topology of these complexes depends on the molecular dimensions of the guest. We have also shown that "inert" zinc porphyrins can be replaced by cobalt porphyrin, allowing the evolution from structural to functional models of heme-proteins.

Supporting Information Available: Experimental procedures, conductometric titration, molecular simulation of **1·2**, ^{1}H NMR spectra for **1c·2** formation and 4-MePyr and caffeine complexation, and an O_2 transport graphic. This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) Magnitude of the shift is consistent with that of the ortho proton of pyridine complexed by a Zn−porphyrin−cyclophane receptor ($\Delta\delta \sim 0.45$ ppm); Benson, D. R.; Valentekovich, R.; Knobler, C. B.; Diederich, F. *Tetrahedron* **1991**, *47*, 2401−2422.

(17) Collman, J. P.; Brauman, J. I.; Doxsee, K. M.; Halbert, T. R.; Hayes, S. E.; Suslick, K. S. J. *J. Am. Chem. Soc.* **1978**, *100*, 2761−2766.