Communications to the Editor

Cyclophane Hemes. 4. Steric Effects on Dioxygen and Carbon Monoxide Binding to Hemes and Heme Proteins

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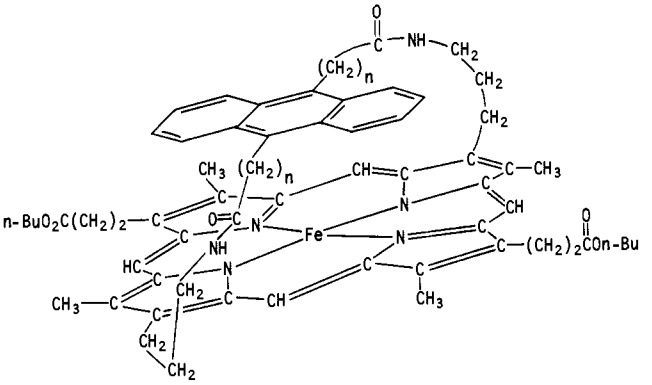
The active sites of heme proteins are closely surrounded by protein side chains in a manner which provides steric hindrance to entering ligands.[1,2] For example, hemoglobin binds isonitriles with affinities decreasing in the order EtNC > *i*-PrNC > *t*-BuNC,[3] a series also found in hindered model compounds.[4a] Additionally, the observations that the Fe–OO bond is bent from the heme perpendicular in both heme proteins and unhindered model compounds[5] whereas such distortion occurs only in heme proteins for Fe^{II}–CO[6-9] groups seem to support the suggestion[10] that the proteins reduce the ratio of CO binding to O_2 binding through distal side steric effects.[2,11,12] Using two new cyclophane hemes having different amounts of steric hindrance to ligand binding, we find that CO and O_2 are subject to the same magnitudes of steric effects and are thus not differentiated by distal side steric effects.

Comparisons of affinities of CO and O_2 for simple unhindered hemes with those of heme proteins have led to conflicting conclusions concerning distal steric effects.[11,13-16] Such comparisons are subject to uncertainties because dioxygen affinities are sensitive to solvent polarity,[17] and both carbon monoxide and dioxygen affinities are affected by structural changes which could preferentially stabilize either the 4-, 5-, or 6-coordinated iron.[16,18] For this reason the relative affinities $K_B^{CO}/K_B^{O_2}$ of CO and O_2 cannot, by themselves, be offered as evidence for steric differentiation of CO and O_2. This ratio can be changed from 5000 to about 300 by merely changing the solvent.

However, distal side steric effects on carbon monoxide and isonitrile binding are characterized in both hemoproteins[2,19] and model systems[4] by a decrease in association rates. Therefore it is important to characterize model systems by kinetic methods.

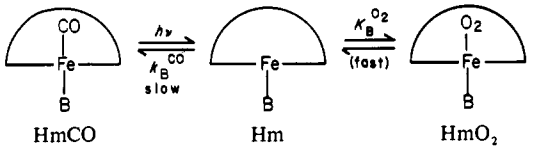
In order to provide definitive evidence concerning steric effects on O_2 binding we have synthesized two heme cyclophanes, **1**[4] and **2**, which differ only by the two CH_2 groups in the connecting



1, n = 1; 2, n = 2

chains and have studied their kinetics and equilibrium properties in benzene where the solvent and the cyclophane bridge would provide similar environments.

The anthracene heme-6,6-cyclophane (**1**)[4] and the anthracene heme-7,7-cyclophane (**2**) were synthesized by the methods previously described,[4b] using anthracene-9,10-diacetic acid and anthracene-9,10-dipropionic acid, respectively. The kinetics of CO and O_2 reactions were determined by our standard flash photolysis procedures[13,20] except for the O_2 reaction with **1** which, because of low O_2 affinity and rapid O_2 dissociation, required that the relaxation from HmO_2 to HmCO (k_{obsd}) be treated as a fast equilibrium followed by slow reaction with CO.



HmCO Hm HmO_2 (1)

$$k_B^{CO}/k_{obsd} = K_B^{O_2}(O_2) + 1 \quad (2)$$

To prevent imidazole binding under the cyclophane cap, the bulky 1,5-dicyclohexylimidazole (DCI),[21] prepared by the method of van Leusen et al.,[22] was used with **1** and **2**.

A plot of $k_B^{CO}(CO)$, the observed pseudo-first-order rate constant without dioxygen, divided by $k_{obsd}(CO)$, the pseudo-

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Table I. Kinetics of Reaction of Hemes with O_2 and CO at 20 °C in Benzene[a]

	k_B^{CO}, M^{-1} s^{-1}	$k_B{}^{-CO}$, s^{-1}	$k_B^{O_2}$, M^{-1} s^{-1}	$k_B{}^{-O_2}$, s^{-1}	$K_B^{O_2}$, M^{-1}	K_B^{CO}, M^{-1}	$K_B^{CO}/K_B^{O_2}$
chelated protoheme	1.1×10^7	0.025[b]	6.2×10^7	4200	1.5×10^4	4×10^8 [c]	27000
chelated mesoheme[d]	8×10^6	0.05[b]	5.3×10^7	1700	3×10^4	2×10^8 [c]	5300
7,7-cyclophane **2**[e]	6×10^6	0.05[f]	6.5×10^7 [g]	1000	6×10^4	1.1×10^8 [h]	1800
6,6-cyclophane **1**[e]	3×10^4	0.05[f]	1×10^5 [g]	800[i]	120	6×10^5 [j]	5000

[a] Rates or equilibria in pressure units were converted to concentration units by using the solubilities of 1.2×10^{-5} M $torr^{-1}$ for O_2 and 9.97×10^{-6} M $torr^{-1}$ for CO.[20b,23] [b] Reference 24. [c] Obtained from $k_B^{CO}/k_B{}^{-CO}$. [d] Solvent toluene–methylene chloride (90:10) except for $k_B{}^{-CO}$ which was measured in $MeOH/H_2O$.[16] Dissociation rates of chelated protoheme are the same in $MeOH/H_2O$ as in toluene.[24] In benzene $k_B^{CO} = 1 \times 10^7$ M^{-1} s^{-1}. [e] The base is 1,5-dicyclohexylimidazole (DCI), 1.2 M for **1**, and 0.03–0.08 M for **2**. [f] Calculated from k_B^{CO}/K_B^{CO}. [g] Observed directly. [h] Obtained by competitive titration against 1-methylimidazole ($K_B^{CO} = 1.1 \times 10^8$ M^{-1}) or *t*-BuNC ($K_B^{CO} = 0.9 \times 10^8$ M^{-1}). [i] Calculated from $k_B^{O_2}/K_B^{O_2}$. [j] Using 1-methylimidazole (0.02 M) as proximal base, the K_B^{CO} was also 6×10^5 M^{-1}.[4a] The kinetic data for **1** are less accurate (±20%) than are those for chelated hemes as a result of difficulties in avoiding the base dissociation mechanism[16] for CO reactions in this hindered compound.

first-order rate constant in the presence of dioxygen, against dioxygen concentration according to eq 2 gives a slope of 120 $M^{-1} = K_B^{O_2}$ for **1**. Prior to this slow return (k_{obsd} = 10–30 s^{-1}) in the presence of dioxygen, a fast process was observed at both 408 and 428 nm, corresponding to the change from **1**–DCI to a mixture of **1**–DCI and (DCI)–**1**-O_2. The observed rate constant was 1560 s^{-1} for return to the equilibrium which, at 0.008 M O_2, results in $HmO_2/Hm = 0.96$. Since $k_{obsd}^{O_2}$ (s^{-1}) = $k_B^{O_2}(0.008) + k_B{}^{-O_2}$ and $k_B{}^{-O_2} = k_B^{O_2}/120$, these results indicate that $k_B^{O_2} = 1.0 \times 10^5$ M^{-1} s^{-1}. Kinetics of reactions with the higher affinity **2** and for chelated protoheme were determined as previously described.[20] These and other pertinent kinetic parameters for **1**, **2**, and chelated hemes are shown in Table I.

We previously observed that **1** binds the first 1-methylimidazole with about the same affinity as does deuteroheme dimethyl ester,[4a] indicating that **1** (and, by inference, **2**) is not subject to steric distortional effects such as those seen in capped hemes.[25] Secondly, the similarities of **2**–DCI and chelated mesoheme in their ligation kinetics and equilibria assure us that the cyclophane bridge itself is not making large changes in either the heme geometry or the heme environment relative to that in benzene solvent. We therefore conclude that our kinetic comparisons of **1**, **2**, and chelated protoheme (or chelated mesoheme) in benzene are valid tests of steric effects. Considering the differences in the proximal bases and the slight differences in solvent in the measurements of kinetic parameters for **2** and chelated mesoheme, their behavior toward CO and O_2 can be considered almost identical. This means that **2** displays little or no steric effect toward O_2 or CO (less than a factor of 2). However, **2** is capable of distal side steric effect as illustrated by its low affinity (8×10^4 M^{-1}) for *t*-BuNC (in the presence of DCI) compared to chelated protoheme (1.7×10^8 M^{-1}).[26]

Comparison of the binding to **1** with that to chelated mesoheme suggests that K_B^{CO} decreases by a factor of about 330 whereas $K_B^{O_2}$ decreases by 250 as a result of the steric effect. A similar comparison of **2** with **1** reveals a 180-fold decrease in K_B^{CO} and a 500-fold decrease in $K_B^{O_2}$. Although one comparison reveals a larger steric effect for CO and the other reveals a larger steric effect for O_2, the differences are small. What seems to be clear is that, in these models, there are large steric effects on O_2 and CO binding and no steric differentiation favoring O_2 over CO in their heme affinities.[27]

The decrease in CO and O_2 affinities in changing from **2** to **1** is seen to result from a decrease by a factor of 200 and 600, respectively, in the association rates, with negligible change in dissociation rates. That both O_2 and CO display this steric effect exclusively in association rates indicates that distal side steric effect is a dynamic effect in the unligated state rather than a static effect in the ligated state.[26] Furthermore, the kinetic criteria for distal side steric effects on CO and RNC[26] are, by these data, extended to O_2 as well.

It is interesting to note that the carbon monoxide stretching frequencies in the complexes 1-methylimidazole–**1**–CO and 1-methylimidazole–**2**-CO are 1975 and 1966 cm^{-1}, respectively, in chloroform.[28] This indicates that steric hindrance does not reduce ν_{CO} as often suggested but tends to cause an increase, in agreement with the conclusions of Jones et al.[29] These results are inconsistent with the proposal that steric effects decrease the CO stretching frequency.[15]

There do seem to be steric effects in the binding of both CO and O_2 to heme proteins. Wittenberg et al. observed reduced association rates for both CO and O_2 in their reactions with horseradish peroxidase (HRP) and noted that the values of $k_B^{O_2}/k_B^{CO}$ for HRP are about the same as those in several other hemoproteins having differing affinities.[30] We therefore conclude that steric effects on CO and O_2 are similar in heme proteins as well as in these models and that the cyclophane compounds mimic this aspect of hemoprotein reactivity rather well.

Myoglobin has about 5–8 times lower values of $(K_B^{CO}/K_B^{O_2})$[16,31a,b,32-34] and (K_B^{CO}/K_B^{NO})[31c,35] than does R-state hemoglobin, and this has been interpreted in terms of steric differentiation.[16,35] The $k_B^{O_2}/k_B^{CO}$ values are 5–10 and 25–30 for R-state hemoglobin and myoglobin, respectively.[30,33,34] It thus appeared that myoglobin favors O_2 over CO by a steric factor of about 5–8. Although there might be steric effects for the distal imidazole which are not duplicated by the cyclophane models, there are other factors, such as removal of (or hydrogen bonding to) the proximal imidazole proton, which could preferentially reduce CO binding. Both the lowered CO stretching frequency and red-shifted CO Soret absorption are consistent with this H-bonding hypothesis.[14] Additionally, the distal imidazole in myoglobin is reported to be hydrogen bonded to oxygen in the dioxygen complex but not in the CO complex.[36]

The present results provide strong evidence that CO and O_2 are not differentiated by steric effects. These results, and the fact

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that R-state hemoglobin shows the same kinetics, equilibrium, and binding enthalpies for O_2 and CO as does chelated protoheme,[13] indicate that such differentiation is not a significant factor in hemoglobins and related dioxygen-transporting heme proteins.

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Nonbonding Steric Effect on CO and O_2 Binding to Hemes. Kinetics of Ligand Binding in Iron–Copper Cofacial Diporphyrins and Strapped Hemes

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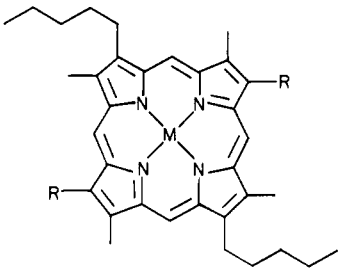
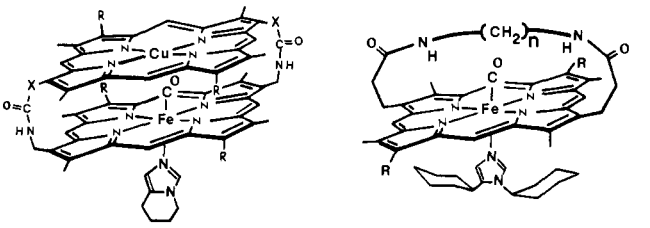
X-ray crystallography showed that the structures of carbon monoxide liganded hemoglobins (Hb) and myoglobins (Mb) exhibit a bent or tilted FeCO linkage with respect to the porphyrin ring,[1-5] whereas in heme model compounds the FeCO bond is linear and perpendicular to the heme plane.[6,7] The origin of the distorted configuration in the proteins is attributed primarily to nonbonding steric interactions of the axial ligand with residues at the distal side. An assumption is made that ligands such as O_2 and NO, which preferentially form bent complexes, should encounter less steric hindrance when bound in the heme pocket.[8,9] It has been proposed that in Hb and Mb, the distal steric effect would decrease the affinity ratio of CO vs. O_2 and is responsible for the detoxification of CO poisoning in respiratory systems.[10-13] A comparison of ligand binding constants of proteins and model compounds often shows that many heme models have a larger CO vs. O_2 affinity ratio (M value) than the proteins. However, such a comparison does not necessarily constitute a correlation between the distal steric effect and affinity as the ligand binding constants of heme models can be drastically altered by medium effects.[14,15] Indeed, Traylor and co-workers have shown that a 5-coordinate protoheme-imidazole model binds both O_2 and CO in aqueous suspension with equilibrium and kinetic parameters almost identical with R-state isolated hemoglobin chains.[14-17] In other cases, for example, T-state hemoglobin and notably myoglobins have very small M values which cannot be duplicated with simple heme compounds.

It is therefore of importance to examine the steric effects on ligand affinity using synthetic models equipped with varying degrees of steric hindrance at the distal side. Several porphyrin models of this kind have been prepared[18-20] and recently an iron complex with a bent CO has been shown,[21] but kinetic rates of ligand binding to the hindered hemes are not available. We wish to report the equilibria and kinetic rates of CO and O_2 binding to two hindered heme systems. One is mixed metal cofacial diporphyrins in which an inert copper porphyrin[22] is tightly linked to the heme, thereby providing a compression from above to the coordinating ligand. The second system is iron cyclophane porphyrins where a hydrocarbon chain is strapped across one face of the heme. Depending on the chain length, the strap would mostly exert a side-way shearing strain to the gaseous ligand.



1, R = CH_2CH_2COCl; M = Cu
2, R = CH_2CH_2COCl; M = 2H
3, R = CH_2COCl; M = Cu
4, R = CH_2NH_2; M = 2H
5, R = CH_2NHAc; M = Fe

Cofacial diporphyrins have been synthesized by coupling porphyrin diamines with diacid chlorides under high dilution conditions.[23] Thus reactions of **1** and **4** and **3** and **4** in CH_2Cl_2–pyridine afforded the copper-free base dimer 5 and dimer 4, respectively. Insertion of iron was accomplished by using the ferrous sulfate method.[24] The strapped hemes 13, 14, and 15 were synthesized by reacting **2** with 1,5-diaminopentane, 1,6-hexanediamine, and 1,7-diaminoheptane, respectively, followed by iron insertion. All porphyrins were characterized by visible,



R = n-pentyl

Fe-Cu-4 X=(CH_2)
Fe-Cu-5 X=$(CH_2)_2$

FeSP-13 n=5
FeSP-14 n=6
FeSP-15 n=7

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