Journal of Macromolecular Science, Part A
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597274

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To cite this Article Chang, C. K. , Ward, Brian , Young, Richard and Kondylis, Michail P.(1988) 'Fine Tuning of Heme Reactivity: Hydrogen-Bonding and Dipole Interactions Affecting Ligand Binding to Hemoproteins', Journal of Macromolecular Science, Part A, 25: 10, 1307 — 1326
To link to this Article: DOI: 10.1080/00222338808053423
URL: http://dx.doi.org/10.1080/00222338808053423

J. MACROMOL. SCI.—CHEM., A25(10 & 11), pp. 1307-1326 (1988)

FINE TUNING OF HEME REACTIVITY: HYDROGEN-BONDING AND DIPOLE INTERACTIONS AFFECTING LIGAND BINDING TO HEMOPROTEINS

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ABSTRACT

Heme reactivity in hemoproteins is governed by the microenvironment near the ligand binding site. In order to quantify polarity effects on heme ligand binding, the kinetics of O_2 and CO binding have been measured for a series of synthetic heme models equipped with a range of groups of varying dipole moments positioned near the heme coordination site. For hemes with polar aprotic groups, both O_2 *on* (k') and *off* rates (k) are found to be dependent on the dipole moment. For model systems containing protic groups, the O_2 *off* rate is substantially reduced due to hydrogen bonding with the coordinated O_2. The hydrogen-bonding stabilization is estimated to be 0.7 and 1.6 kcal/mol for an alcohol and a secondary amide, respectively. CO binding displays little correlation with a polarity effect; instead it seems to depend upon the size and position of the polar group.

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INTRODUCTION

The active site of hemoproteins is a highly functional architecture in which the reactivity of the heme group is controlled by the pocket structure. In the archetypal hemoglobin and myoglobin, the heme-ligand binding reaction is governed by electronic and steric effects originated from the proximal histidine as well as by the microenvironment surrounding the ligand binding site [1]. The latter includes protein shielding of the heme iron, steric distortion of the coordinated ligand, dipole-dipole, and hydrogen bonding interactions. Following the discovery in 1973 that iron porphyrins can be made to reversibly bind dioxygen without oxidation in solution [2-4], model compounds have been used extensively to probe the various factors that may control the heme properties [5]. This paper reports our attempt to measure quantitatively the polarity effects on O_2 and CO binding.

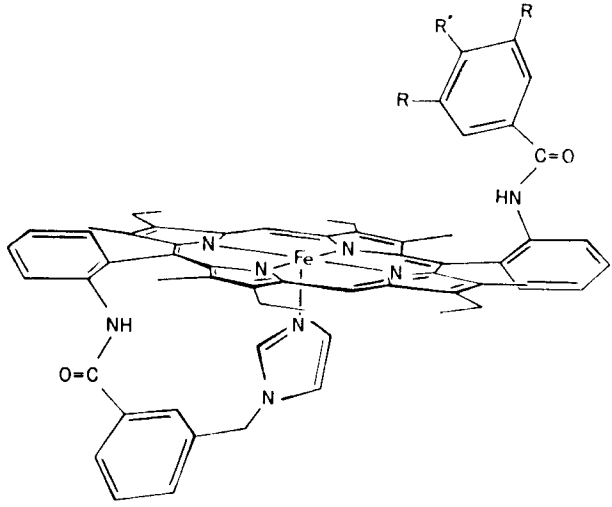
That the distal histidine may form hydrogen bonding to the coordinated oxygen and thereby stabilize FeO_2 is well supported by x-ray evidence [6]. Previous model studies have unmistakably demonstrated that O_2 binding to hemes is very sensitive to solvent polarity, owing to the dipolar nature of bound O_2, whereas CO binding seems to be less affected [7]. While several studies on solvent effects have been reported [7-9], the influence of local polarity at the heme coordination site remains difficult to measure quantitatively because of the large number of variables involved when cross-comparing synthetic models of different structure and different solvation shell. In addition, solvent effects alone cannot mimic the action of a protein environment since the protein pocket acts as an "ordered solvent" that maximizes the effect. There is also a question about H-bonding versus dipole-dipole interaction; How important is each effect? For instance, neutron diffraction studies on oxyMb [10] and oxyHb [11] have confirmed that the terminal oxygen of FeO_2 is within hydrogen-bonding distance of the distal imidazole proton, yet the kinetics of oxygen binding to model hemes in *protic* solvents [7, 8] do not show large differences from those in polar *aprotic* media. In order to provide answers to these questions, synthetic heme models equipped with a range of polar groups situated at similar positions from the heme center have been synthesized. Here we report the kinetics of O_2 and CO binding to such heme models and to compounds capable of providing hydrogen bonding to the bound ligand. By studying the O_2 and CO binding of these compounds in an identical environment (i.e., in toluene), we can quantify distal polarity effects where the heme cavity may be regarded as containing a dominant dipole and/or H-bond donor in a hydrophobic environment. This study thus determined, for the first time, the individual contribution of dipole-dipole versus H-bonding interactions in stabilizing the Fe-O_2 complex.

EXPERIMENTAL

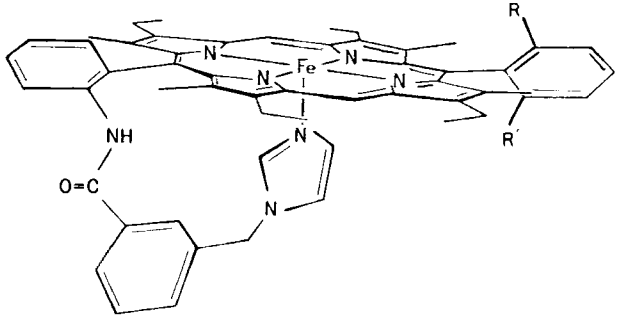
The synthesis and characterization of Compounds **1a-g** and **2a,b** has been reported [12]. Toluene was purified by stirring with several changes of concentrated H_2SO_4, followed by drying over anhydrous sodium carbonate and distillation from lithium aluminum hydride just prior to solution preparation. Sample solutions for kinetics and CO titrations were prepared by dissolving the ferric compounds in ~4 mL of toluene (10^{-5} *M*) containing 10^{-4} *M* benzophenone. The solutions were degassed in an 80-mL tonometer by freeze-pump-thaw cycles. The hemin chlorides were reduced by photolysis according to the method previously described [13]. Flash photolysis was carried out with either a xenon photographic flash gun or a flash-lamp-pumped dye laser with rhodamine 6G dye. Decay constants were calculated from transmittance versus time plots at 432 nm (5-coordinate heme disappearance), 410 nm (oxyheme appearance), or 418 nm (CO-heme appearance) recorded on an oscilloscope. CO and O_2 concentrations ranged from 5×10^{-5} to 6×10^{-3} *M* and from 5×10^{-4} to 6×10^{-3} *M*, respectively. CO association rate constants (l') were calculated from plots of the observed pseudo-first-order rate constants versus CO concentration: these plots typically had correlation coefficients of 0.998 to 1.000 and varied between experiments by less than 5%. O_2 association rate constants (k') were calculated from similar plots with correlation coefficients not less than 0.95 (variance between experiments <7%). Oxygen affinities were determined by CO competition measurements and calculated according to the Gibson equation [14]:

$$\frac{1}{R} = \frac{1}{k} + \frac{K(O_2)}{l'(CO)}, \tag{1}$$

where $l'(CO)$ is the observed pseudo-first-order rate constant determined before the introduction of O_2. O_2 equilibrium measurements had correlation coefficients 0.992-1.000 and varied between experiments by 2% or less. Oxygen dissociation rate constants were calculated from the observed oxygen association rate and equilibrium constants. carbon monoxide affinities were determined by direct titration of the heme with a gas mixture containing 0.072% CO in argon. CO dissociation rate constants were calculated from $l = l'/L$.



1a R = H R′ = t-Bu
b R = CH_2OMe R′ = H
c R = CH_2OH R′ = H
d R = $CO_2C_4H_9$ R′ = H
e R = $CONHC_4H_9$ R′ = H
f R = $CONEt_2$ R′ = H
g R = $CONi\text{-}Pr_2$ R′ = H



2a R = H R′ = $NHCOCH_3$
b R = $NHCOCH_3$ R′ = H

RESULTS AND DISCUSSION

Oxygenation Kinetics

The model system we chose to study consists of a series of well-characterized 5-coordinate heme compounds differing principally in the dipole moment of the phenyl substituent above the O_2 binding site (Compounds **1b-g**). The synthesis and the advantages of having the imidazole base linked via a benzamide group have been described previously [12]. 3,5-Substituted benzamides were chosen so that the orientation of the meta-substituents would be inconsequential. Monosubstituted benzene dipole moments [15] were included in the tabulation of O_2 and CO binding kinetic rates (Table 1) to provide a scale of the dipole strength of these substituents.

From Table 1 it is apparent that the oxygen association rates (k') decrease as the distal substituents become increasingly polar. With the exception of **1c**, **e**, and **g**, the O_2 dissociation rates (k) also progressively decrease. Assuming that the reaction between O_2 and the heme models can be represented by the diagram shown in Fig. 1, it is not surprising that k and k' would correlate with the dipole moment of the substituent groups. According to the Kirkwood-Laidler-Eyring model [16], a reaction between two dipolar species A and B in a solvent of dielectric constant c may be described by:

$$\ln k = \ln k_0 - \frac{(\epsilon - 1)}{kT(\epsilon - 1)}\left[\frac{\mu_A{}^2}{r_A{}^3} + \frac{\mu_B{}^2}{r_B{}^3} - \frac{\mu_\ddagger{}^2}{r_\ddagger{}^3}\right], \qquad (2)$$

where μ_A, μ_B and $\mu_\ddagger$ are the dipole moments of the reactants and activated complex, respectively; and r_A, r_B, and $r_\ddagger$ are the corresponding molecular radii. Since the net dipolar change upon oxygenation, as felt by the solvent, can be regarded as the sum of μ_{FeOO} and the distal side dipoles, the above equation implies that $\ln k'$, as well as $\ln k$, can be expressed by a quadratic equation in terms of dipole moment of the substituent group. Indeed, a reasonable fit can be obtained (Eq. 3 and Fig. 2A). Because of the uncertainties involved in determining the actual dipole moment, no special physical meaning is ascribed to the coefficients of Eq. (3) other than to indicate that the oxygenation process can be affected in a predictable manner by a nearby dipole.

$$\ln k'_{calc} = 0.092\ (\pm 0.005)[\mu]^2 - 0.75\ (\pm 0.014)[\mu] + 18.0\ (\pm 0.1) \qquad (3)$$

$$\ln k_{calc} = -0.015\ (\pm 0.03)[\mu]^2 - 0.26\ (\pm 0.16)[\mu] + 9.7\ (\pm 0.3) \qquad (4)$$

TABLE 1. CO and O_2 Binding Constants of Diphenyl Hemes with Groups of Varying Polarity Situated Near the Ligand Binding Site[a]

Compound	μ_g[b]	k', $M^{-1} \cdot s^{-1}$	k, s^{-1}	$P_{1/2}{}^{O_2}$ torr	l', $M^{-1} \cdot s^{-1}$	l, s^{-1}	$P_{1/2}{}^{CO}$, torr	M[c]
1a 4-*t*-Butyl	0.52	4.7×10^7	15 500	33	2.5×10^6	0.14	0.0057	5 800
1b 3,5-CH_2OMe	1.3	2.6×10^7	9 900	38	1.1×10^6	0.028	0.0075	15 000
1c 3,5-CH_2OH	1.7	2.3×10^7	2 900	13	1.2×10^6	0.090	0.0075	1 700
1d 3,5-CO_2nBu	1.9	2.2×10^7	11 000	48	1.5×10^6	0.13	0.0086	5 600
1e 3,5-CONHnBu	3.6	1.3×10^7	300	2.3	1.3×10^6	0.042	0.0032	720
1f 3,5-$CONEt_2$	3.8-3.9	1.4×10^7	4 750	34	0.59×10^6	0.048	0.0081	4 200
1g 3,5-$CONiPr_2$	3.8-3.9	1.3×10^7	9 300	72	0.47×10^6	0.053	0.011	6 500

[a] At 20-22°C in toluene.
[b] Reference 15.
[c] $P_{1/2}{}^{O_2}/P_{1/2}{}^{CO}$.

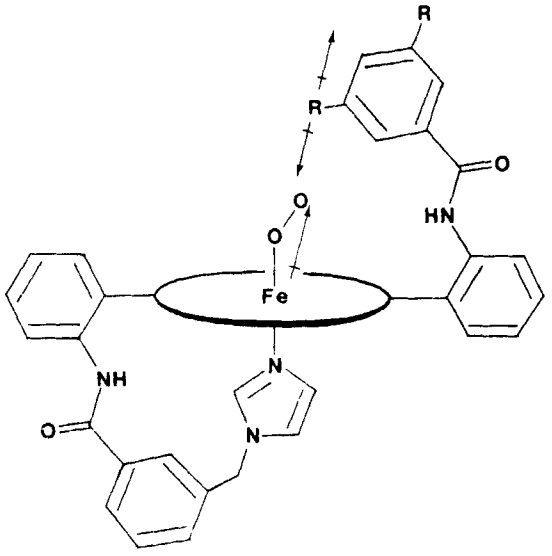


FIG. 1. Relative orientation of the FeOO dipole and the dipole of a *meta*-substituted benzamide.

When ln k was plotted against μ_R, considerable scattering was encountered (Fig. 2B). On the basis of solvent effects, it is apparent that the large deviation found for the 3,5-di-*n*-butyl amide and 3,5-dibenzylic alcohol substituted hemes is caused by hydrogen bonding to Fe-O_2. Also from solvent studies described below, it appears that the bulkier isopropyl group of **1g** may suffer from steric hindrance resulting in a less effective dipolar interaction. If we exclude these compounds, the remaining data points can then be fitted to Eq. (4). The very small data set, however, renders the equation less meaningful. In fact, ln k could have been fitted well to a linear dipole-dipole interaction.

To substantiate that the perturbation on k and k' is indeed due to local polarity difference and H-bonding, kinetics of O_2 and CO binding were measured for three amide compounds (**1e, f, g**) as a function of the concentration of *N*-dimethylacetamide (DMA), as well as *N*-methylacetamide (MA), in toluene. Figures 3A and 3B give plots of the reciprocal rate of O_2 displacement by CO ($1/R$ in Eq. 1) vs [DMA] and [MA] for the three above-mentioned compounds. Since k' and l' were separately determined to be little affected ($<11\%$) by the presence of DMA or MA in the range of 0.1 to 1.0 M, any changes in $1/R$ thus reflect directly the variation of k. As shown in Fig. 3(A),

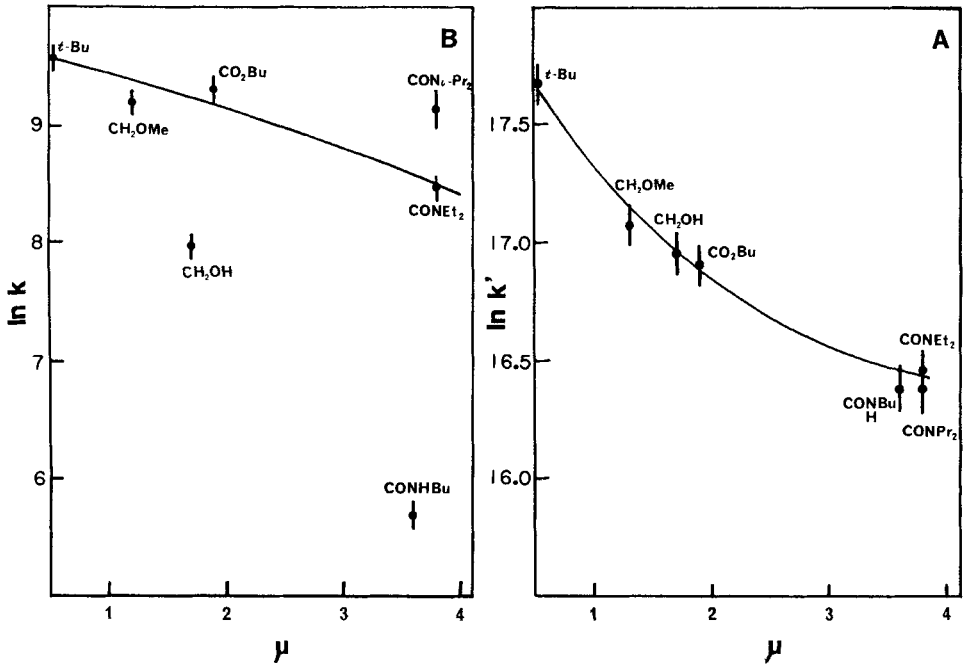


FIG. 2. (A) Correlation between observed oxygen association rate and dipole moment of benzamide substituents. (B) Correlation between observed oxygen dissociation rate and dipole moment of benzamide substituents.

this rate is invariant within experimental error for 3,5-$CONHC_4H_9$ (**1e**) and 3,5-$CONEt_2$ (**1f**), while $1/R$ of 3,5-$CONiPr_2$ (**1g**) increases with increasing [DMA]. That is, the O_2 dissociation rate of **1g** can be reduced by the presence of polar cosolvent. We interpret this an evidence that the steric bulk of the diisopropyl amide does not allow as strong a dipole-dipole interaction as the diethyl amide. Indeed, CPK models suggest that the distance and relative orientation between the FeO_2 dipole and the amide dipole would be restricted by large substituents.

With the protic MA (Fig. 3B), the presence of the cosolvent does not change the rate for **1e** but produces a visible effect of increasing $1/R$ for **1f** and **1g**. Furthermore, the change is more rapid for **1g** than for **1f**. From these observations we may conclude: 1) That the O_2 dissociation rate for the secondary amide **1e** is much slower than for other amides is the result of H-bonding be-

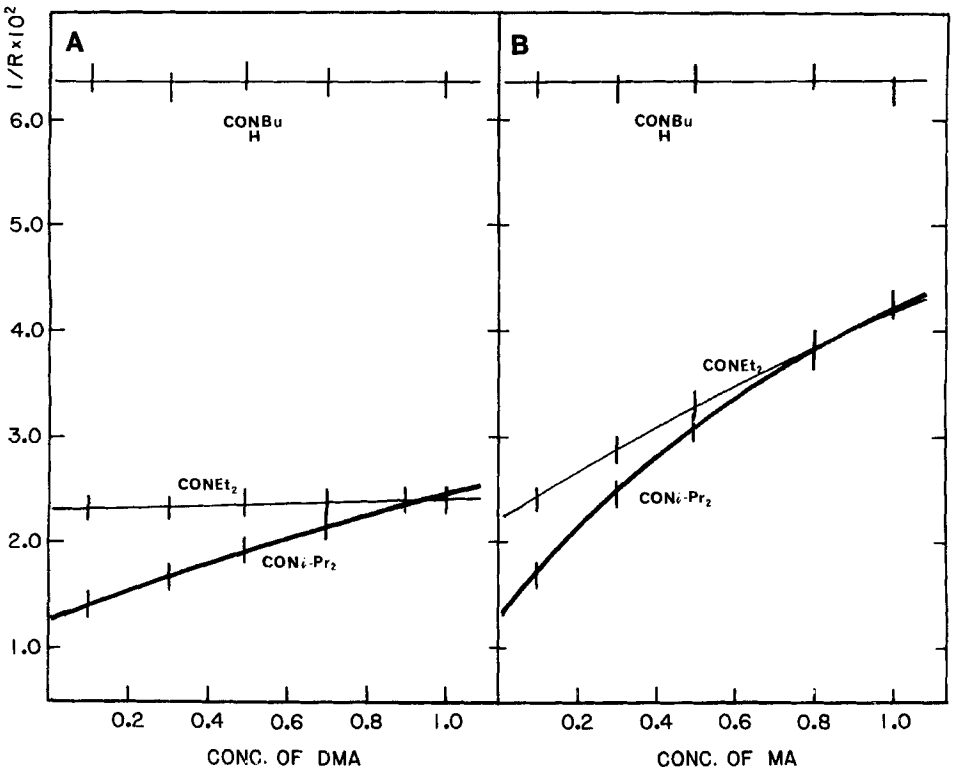


FIG. 3. (A) Effect on the O_2 displacement rate R upon addition of *N,N*-dimethylacetamide to the toluene solvent, $[O_2]/[CO] = 5$ for **1f** and **1g** and 2 for **1e**. (B) Similar effect upon addition of *N*-methylacetamide, under the same condition.

tween FeO_2 and amide N–H proton. In fact, this intramolecular H-bonding is so effective that it is not perturbed by exogenous proton donors or medium effects. 2) The smaller tertiary amides such as **1f** have a reasonably good dipolar interaction with the FeO_2, which is relatively independent of external dipoles but can be affected by H-donors. 3) Bulkier tertiary amides may suffer from steric hindrance and only produce a weak dipolar interaction with the coordinated ligand.

The deviation of k of the alcohol **1c** from the expected dipolar contribution in Fig. 2(B) is deemed also a result of H-bonding. The most striking fea-

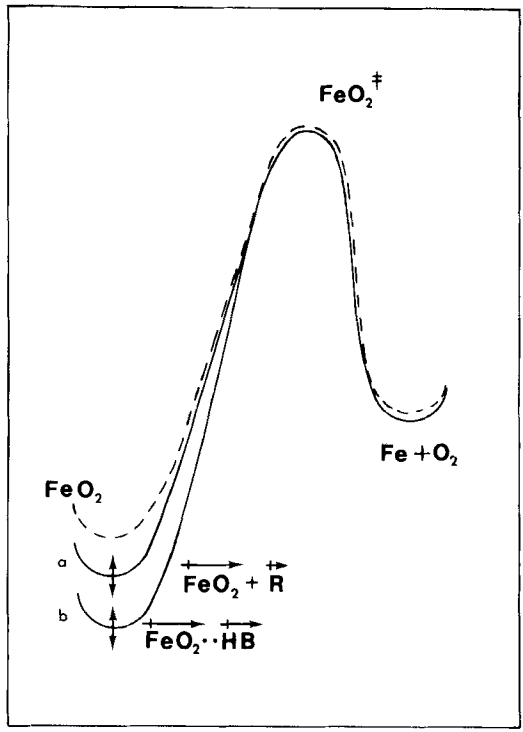


FIG. 4. Schematic representation of reaction coordinates of heme oxygenation. Hypothetical unperturbed coordinate (- -), in the presence of an interacting dipole (a) and hydrogen-bonded oxyheme complex (b).

ture revealed by Fig. 2(A) and 2(B) is that the presence of H-bond donating substituents only generates large perturbations on the O_2 dissociation, but not on the association rate constants, suggesting that H-bonding occurs subsequent to a rate-limiting process for association. A simplified reaction coordinate for heme oxygenation is shown in Fig. 4. The O_2 association rates depend on dipole interactions in the transition state while dissociation rates depend on dipole interactions in both the transition state and oxyheme complex. The stabilization of oxyheme by H-bonding is estimated to be 0.6-0.8 and 1.6 kcal/mol for **1c** and **1e**, respectively. These are the $\Delta\Delta G$ values for **1e** relative to the diethyl amide **1f**, and **1c** relative to the methyl ether **1b** or butyl ester **1d** where $\Delta G = -RT \ln K$.

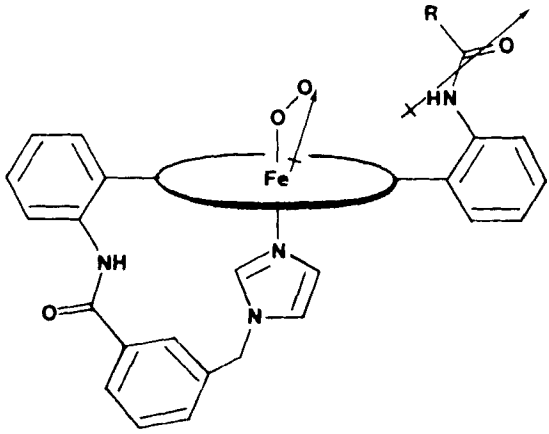


FIG. 5. Scale drawing of the relative orientation and separation of the FeOO dipole and an *o*-anilide dipole.

While the above results clearly demonstrate the influence of polar substituents on the unique benzamido group hovering over the coordination site, there is another dipole present in the synthetic heme structure. It is the *o*-anilido group next to the meso position (Fig. 5). The functionalization of tetraphenylporphyrin family compounds via the *o*-amino groups has become a favorite choice in contemporary heme model designs beginning with Collman's pioneering work of the picket-fenced porphyrin [3a]. This amide group is located about 4 Å away from the FeO_2, as shown by the x-ray structure [17], but its importance in modulating the heme–oxygen binding was not realized until recently. Momenteau and Lavalette [18] were the first to show the dramatic difference in oxygen binding to a heme equipped with anilido groups versus a heme with ether groups. While these workers used laboriously synthesized strapped porphyrins and pyridine axial base, the same principle can be more effectively illustrated by comparing the two simple rotomers **2a** and **2b** in which the polar acetamido group is either on the same (*trans*) or the opposite (*cis*) side of the O_2/CO binding site. The O_2 and CO rate constants are given in Table 2, along with two other entries from the work of Momenteau and Lavalette [18].

These data suggest that the enhanced O_2 affinity of the *trans*-acetamide relative to the *cis*-acetamide must be due to a favorable interaction of the Fe–OO and amide dipoles, in view of the large variation of k, which increases

TABLE 2. CO and O_2 Binding Constants of Diphenyl Hemes with Remote Polar Groups[a]

Compound	k', $M^{-1} \cdot s^{-1}$	k, s^{-1}	$P_{1/2}{}^{O_2}$ torr	l', $M^{-1} \cdot s^{-1}$	l, s^{-1}	$P_{1/2}{}^{CO}$, torr	M[e]
2a *cis*-Acetamide	3×10^7[b]	38 000	126	2.3×10^6	0.12	0.0054	23 000
2b *trans*-Acetamide	2.6×10^7	4 100	16	1.6×10^6	0.072	0.0045	3 550
Ether strap[c,d]	3.0×10^8	40 000	18.6	6.8×10^7			
Amide strap[c,d]	3.6×10^8	5 000	2	3.5×10^7			

[a]At 20-22°C in toluene.

[b]Due to the low O_2 affinity, high oxygen concentrations were necessary, leading to pseudo-first-order rate constants approaching the limits of detection.

[c]Axial base was a covalently attached pyridine.

[d]Reference 18.

[e]$P_{1/2}{}^{O_2}/P_{1/2}{}^{CO}$.

by 800 to 900% on going from the same-side amide compound to the *cis* acetamide. Furthermore, we believe that this enhanced affinity is derived from a nonbonding dipole-dipole interaction and not from a direct hydrogen bonding. In the crystal structure of FeO_2(TpivPP)(MeIm) [17] there is a four-way statistical disorder of the terminal oxygen atom which bisects the N_{pyr}-Fe-N_{pyr} angle and thus points toward the amide N–H protons. The crystallographic structure also shows that the terminal oxygen to N_{amide} distance is approximately 4 Å, which is too long for effective H-bonding compared with a typical peptide H-bonding distance of 2.8-3 Å (bearing in mind that the van der Waals contact distance is only 3.4 Å) [19]. In a very recent paper, Jameson and Drago [20] suggested that weak H-bonding may occur in such a system. Nevertheless, we argue that, because the O_2 dissociation rate reduction from **2a** to **2b** is of the same order of magnitude as that from **1a** to the tertiary amide **1f**, nonbonding dipole interactions alone can adequately account for the enhanced O_2 affinity of **2b** (ΔG is about 0.5-0.8 kcal/mol). We concur, however, that H-bonding could happen in a tightly strapped system where the amido groups are brought closer to the center of the heme, and there is indeed evidence to support this contention (*vide infra*).

As for the Fe-O-O orientation in relation to the heme coordinates, we notice that in oxyhemoglobin [11, 21] and oxymyoglobin [10, 22] the O-O axis eclipses an N_{pyr}–Fe bond, whereas in picket-fence heme [17] it points toward the meso substituents. The difference may be the result of polar interactions. Inspection of stereoviews of the active sites for oxyHb [11] and oxyMb [10] reveals that an FeO_2 dipole may be practically aligned with the distal histidine dipole in a head-to-tail fashion besides being hydrogen bonded.

Carbonylation Kinetics

Since free carbon monoxide has a dipole moment (μ_{CO} = -0.112 D [23]), and bond formation between Fe and CO increases the dipole moment of CO [24], it is expected that dipole-dipole interactions play some role in carbonylation kinetics. However, as shown in Fig. 6 and consistent with previous solvent studies [7, 8], we found little correlation between CO kinetics and the magnitude of a substituent dipole moment. For the CO association rate, it appears that electrostatic perturbations are outweighed by nonelectrostatic factors. The variation seems to be related to the size and position of the phenyl substituent.

It has been suggested [25] that perturbation of CO and O_2 binding by steric effects is most pronounced in the association rate constants. Indeed,

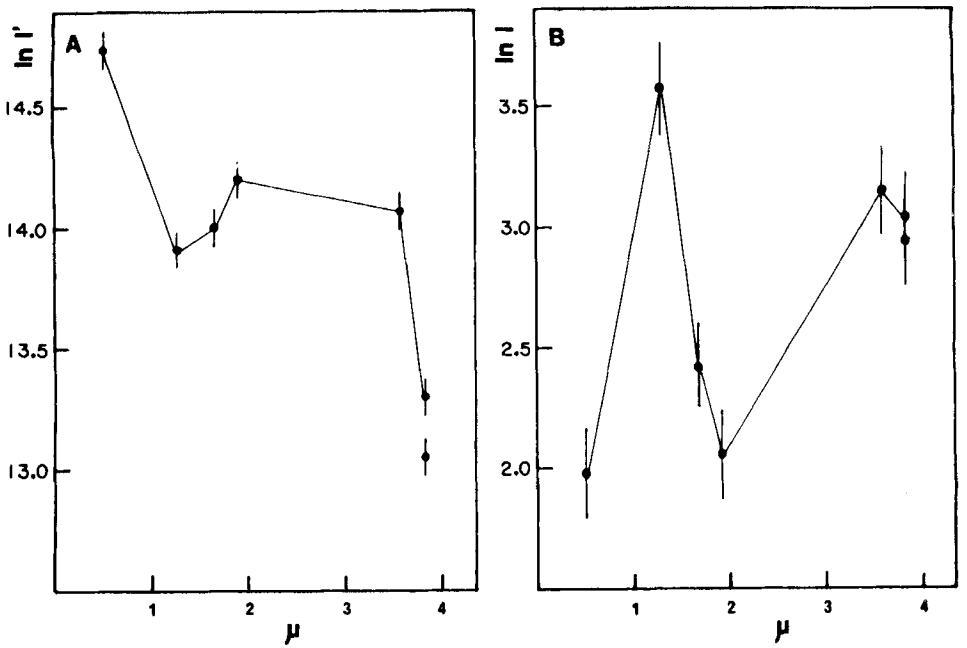


FIG. 6. (A) Plot of the logarithm of CO association rates versus dipole moment of benzamide substituents. (B) Plot of the logarithm of CO dissociation rates versus dipole moment of benzamide substituents.

a comparison of the three amides in Table 1 shows that increasing the size of N substitution regularly decreases l' with almost no effect on l. Oxygenation rate constants, however, remain almost constant. Furthermore, Compounds **1b-d** have very similar CO association rate constants, which differ significantly from that for **1a**. This may be due to the position of substitution on the benzamide. CPK models reveal that, at the heme center, Compounds **1b-d** have a similar steric bulk which is greater than that present in **1a**. The possibility of hydrogen bonding to carbonylated hemoproteins has previously been discussed [26]. It is evident that compounds **1c** and **1e** could provide hydrogen bonding to FeO_2, yet there is no apparent hydrogen bonding effect on carbonylation kinetics. Suslick recently reported that in a "bis-pocket" heme, the CO affinity decreased with increasing solvent polarity [9]. In the absence of kinetic analyses, we cannot offer an explanation for this disparity.

Distal Steric Effect

Recently much effort has been put forth in the synthesis and characterization [27-30] of models useful in testing the distal steric effect hypothesis [25, 31]. Based on structural evidence that the FeCO linkage is bent in Hb and Mb, a distal steric effect that brought about the distortion of the otherwise linear FeCO bond has, by default, been attributed to be responsible for the discrimination of CO binding in Mb and Hb [25]. Despite the simplistic appeal of this proposal, model studies to date [27-30] have failed to conclude that steric distortion alone can account for the ligand binding specificity. It is true that a distal steric effect does modify oxygen and carbon monoxide association rates, but the effect on dissociation rates, primarily for oxygen, is in question. Conflicting results have been obtained for the relationship between oxygen dissociation rates and steric encumbrance [27-30]. From the work presented here, it becomes evident that the lack of a clear-cut answer to steric differentiation may be attributable to dipole-dipole and/or hydrogen bonding variability within a seemingly congruent series of compounds.

For Collman's picket-fence-based compounds, O_2 dissociation rates decrease markedly as the cavity of the ligand binding site shrinks. The reported oxygen dissociation rates are, respectively, 2900, 71, and 9 s^{-1} for $FePiv_3$5CIm, FeMedPoc(MeIm), and FePocPiv(MeIm) [29]. From CPK models it appears that the introduction of the phenyl cap on top of the heme face (pocket hemes) results in pulling of the amide moieties toward the heme center (Fig. 7.). This motion brings about a closer head-to-tail

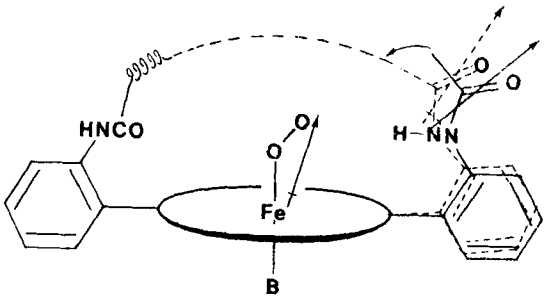


FIG. 7. Variation in the *o*-anilide dipole orientation upon introduction of a tight strap across the heme surface. (—) Unconstrained. (- -) Sterically encumbered model.

dipole-dipole interaction between the amides and FeO_2, relative to Fe picket fence. In fact, for FePocPivP it appears that the amide proton may come close enough (~3 Å) for hydrogen bonding. Indeed, in a system similar to the pocket hemes, Momenteau detected H-bonding occurring between FeO_2 and peripheral amide linkages [31].

The alkyl amide linkages of Traylor's anthracene cyclophane hemes [28] should be flexible enough to rotate, and it is not difficult to find conformations in which the amide dipole aligns with a FeO_2 dipole. In the compound with longer anthryl straps, the amide dipole may become more distant from the heme center. Oxygen dissociation rate constants for 6,6-cyclophane heme (k = 800 s^{-1}) and 7,7-cyclophane heme (k = 1000 s^{-1}) are consistent with this interpretation. Our linear-chain strapped hemes also show this correlation [27], i.e., on shortening the strap we obtained a decrease in oxygen dissociation rate constants: 250, 175, and 130 s^{-1} for FeSP-15, FeSP-14, and FeSP-13, respectively.

Among all studies of sterically hindered models that contain amide linkages, the only inconsistency lies in the O_2 dissociation rate data for our Fe-Cu cofacial diporphyrins [27] where k for Fe-Cu-4 and Fe-Cu-5 is 160 and 91 s^{-1}, respectively. We suggest that the relatively large distance between the amide group and the heme center could render the dipole interaction relatively insignificant and that the reduced k for Fe-Cu-5 could result from an increased accessibility to FeO_2 by polar molecules (imidazole) present in the system. This is not the case with the strapped hemes since the linear hydrocarbon chain provides much less shielding than does the Cu porphyrin cap. Another possibility is that the terminal oxygen atom may weakly coordinate to Cu and, due to the variation in porphyrin-porphyrin distance and conformation [32], such an interaction is not expected to be constant in the two compounds. For the Baldwin-Basolo "capped" porphyrins [30], the absence of amide dipole in the ether strapped compounds would render the system more sensitive to steric influences. It is also possible that an unfavorable dipole-dipole orientation may exist between FeO_2 and the ester groups on the cap. Unfortunately, lacking the essential kinetic data, we are not able to tell which of the four rates was altered most to produce the overall result of discriminating O_2 binding.

We wish to stress that with models thus far available, all attempts to assess the steric differentiation between CO and O_2 binding based upon the affinity ratio M have not been successful since *one cannot ascertain how much change in O_2 dissociation rate comes from pure ligand distortion and how much is due to polarity changes,* let alone the effect of bending and ruffling of the prophyrin plane introduced by the encumbrance of superstructure [33]. Mims

et al. [34] recently presented arguments that a distal steric effect of destabilizing CO is of less importance than the stabilization of Fe-O_2 via hydrogen bonding. From the data presented in Table 1, we clearly demonstrated that M values can be dramatically reduced as the result of H-bonding. If one compares the reduction of M from the chelated protoheme to Hb a-chain and to Mb, M decreases from 560 [8] to 130 [25] to 30-40 [14], respectively. Assuming that the H-bonding occurring in the chelated protoheme-micelle solution can be approximated by **1e**, the H-bond strength increases in the order Mb > Hb > chelated heme with FeO_2 being stabilized by 2, 1, 0.6-0.8 kcal/mol, respectively. As well, nonbonding dipole interactions could add additional discrimination against CO binding, as illustrated by data in Tables 1 and 2, but this contribution will be less prominent in comparison with the direct H-bonding. Nevertheless, in hemoproteins that lack a protic residue near the heme group, this force may well play a dominant role.

This discussion cannot end without commenting on another chief point of controversy, namely: Could a shape-selective binding site differentiate the O_2 versus CO *association* rate? Although both k' and l' are found to change with steric encumbrance, the ratio k'/l' obtained from existing sterically hindered models varies so contradictorily that virtually no useful conclusion can be extracted from these data. Traylor [28b] recently pointed out that most synthetic models control the association kinetics by limiting the accessability of the binding site, and thus the reduced association rate merely reflects a preequilibrium for opening up the site, which is not necessarily related to the final configuration of the coordinated ligand. Still, a suitably designed system with appropriate steric attributes may provide a relevant test for this question. Our present series of model compounds, although not specifically designed to accomplish this feat, appears to be applicable. During the addition of protic cosolvent to 3,5-$CONEt_2$ **1f** and 3,5-$CONiPr_2$ **1g**, k of both compounds should ultimately decrease to a limiting value equal to that of **1e** (Fig. 3B). Assuming k = 300 is the norm for an optimized H-bonded FeO_2, the M values calculated under these conditions are 720, 290, and 210 for, in the order of increasing substituent size, **1e**, **1f**, and **1g**, respectively. Because of the limited data range, we are reluctant to say that the diminution of l'/k' according to steric bulk constitutes a proof for the above question. Nevertheless, this interesting observation does suggest a starting point for the design of other molecules that will more rigorously test the steric effect hypothesis.

CONCLUSION

Our work presents clear evidence showing that dipolar forces and hydrogen bonding can play a significant role in regulating oxygen affinities of heme proteins. While this general conclusion is consistent with previous solvent studies, the use of covalently attached polar groups has offered great advantages in probing the microenvironment of the heme coordination site. We have demonstrated that dipolar forces can produce kinetic and thermodynamic control, and that hydrogen bonding provides an additional path for control of heme oxygenation. In contrast, CO binding to hemes is little affected by distal polar groups.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation. B.W. thanks Amway Corporation for a 1982 summer fellowship.

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Received January 19, 1988