A Kinetic Model for R- and T-State Hemoglobin. Flash Photolysis of Heme–Imidazole–Carbon Monoxide Mixtures

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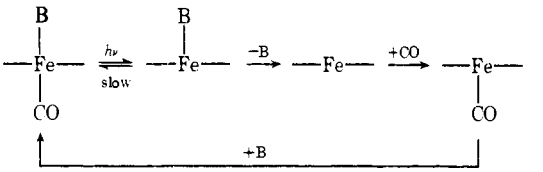
Abstract: The rates of binding of carbon monoxide and imidazoles to ferrous hemes are reported. By flash photolysis of appropriate heme(CO)(Im) mixtures it is possible to determine all ten rate constants relating the five species heme, heme(CO), heme(Im), heme(Im)$_2$, and heme(CO)(Im). Under all conditions, some of the return from heme(Im) to heme(CO)(Im) proceeds via loss of imidazole, followed by addition of CO and subsequent addition of imidazole, the "base-elimination" pathway. Both benzene and aqueous detergent (cetyltrimethylammonium bromide) have been used as solvents; the similarities and differences are discussed. The rates of the heme–CO–imidazole mixtures are compared with those found in heme proteins. The sterically hindered 2-methylimidazole serves as a model for T-state hemoglobin, while the unhindered imidazole or 1-methylimidazole serves as a model for R-state hemoglobin.

Introduction

The mechanism of cooperativity in hemoglobin remains a subject of continuing study.[1] Cooperativity hinges first upon relaying to the other hemes the information that one heme has been ligated, and second upon utilizing this information to alter the reaction rates at those hemes. While the former aspect has now been investigated in some detail, the latter is not well understood at the atomic level. Of the many ways in which the protein might control the rate of reaction of a ligand with the heme nucleus, the most widely accepted is the trigger mechanism proposed by Perutz and co-workers[2] following suggestions of Williams[3] and Hoard.[4] In this proposal the low-affinity (T) state is characterized by a large iron–porphyrin distance induced by the protein pulling on the proximal histidine, whereas the high-affinity (R) state is characterized by a smaller iron–porphyrin distance, resulting from a relaxation of the protein, and a movement of the proximal histidine toward the heme. Extensive kinetic and equilibrium studies of hemoglobin have revealed much about the effects of protein conformational change on the reactivity of the individual iron atoms. For example, a single heme in T-state hemoglobin binds carbon monoxide 60 times more slowly and releases it ten times more quickly than it does when it is in R-state hemoglobin.[5] However, the T-state heme reacts with oxygen only seven times more slowly than does the R-state heme, but releases oxygen about 150 times more quickly.[6] The consequence is that CO and O_2 equilibria show similar R- to T-state changes, but for different reasons.

The difference between the T and R states of hemoglobin is usually expressed in terms of the "strain" of the T state. The word strain has come to have a variety of meanings in hemoglobin discussions, in every case relating to a slow-reacting heme, but referring variously to doming of the porphyrin, to ligand–heme plane interactions, to a stretching of the iron–imidazole bond, and to high-energy protein conformations. Attempts to localize this strain fall generally into four categories. A variety of experimental techniques including X-ray absorption,[7] NMR,[8] Mössbauer,[9] and Raman[10] spectroscopy have probed the heme ring in hemoglobin with little evidence of strain. Calculations have also appeared which correlate the observed structure of the metalloporphyrin with the strain energy of the porphyrin skeleton.[11] In a third approach, cobalt porphyrins and hemoglobins have been used to probe the relation of geometry to cooperativity with conclusions both for[12,13] and against[14] the Perutz mechanism. Finally, hindered bases have been used to model the reactions of the strained hemoglobin T state.[13,15,16] In this case, the binding of a hindered base apparently results in doming of the porphyrin skeleton.

The first evidence for the proposed strain effect in the reactions of simple hemes with gaseous ligands was the report by Rougee and Brault[16,17] that the carbon monoxide binding constant for deuteroheme dimethyl ester was reduced by a factor of approximately 200 when imidazole was replaced by the sterically hindered 2-methylimidazole: $K^{CO}_{2\text{-MeIm}}/K^{CO}_{Im} = 0.0051$. This effect of 2-methylimidazole on equilibria of ligation has recently been extended to oxygen binding of cobalt porphyrins with similar results.[13] We have reported the effects of such strain on the kinetics and equilibria of O_2 and CO binding to a series of hemes bearing a covalently attached base, the "chelated heme" model systems.[15b] Introduction of imidazole–iron strain in three different ways revealed patterns for oxygen binding which paralleled those of R- and T-state hemoglobin and showed that the cooperativity of hemoglobin could indeed be attributed to changes in the strain in the system. However, carbon monoxide binding reacted differently to the introduction of strain. It was shown that strained models added CO in large part not to the five-coordinated heme HB, but rather to the four-coordinated heme H, the "base-elimination" pathway.[15] As a consequence, increasing the imidazole–iron strain increased the rate of reaction of CO with the heme, rather than decreasing it. The amount of this pathway depends upon the relative reactivities of the four- and five-coordinated hemes, and on the equilibrium between them. It has been found that even unstrained heme–imidazole–CO mixtures react at least partly by this mechanism. Increasing the local concentration of base and reducing its off rate by synthesis of a strain-free "chelated heme" results in straightforward direct association of CO. This has led to increasing adoption of "chelated heme" model compounds for quantitative studies.[13,18]



Although the straightforward association of CO with HB cannot be observed directly in heme–base–CO mixtures, we considered it possible to derive the on and off rates of CO with HB by extrapolation, in a manner similar to that used by

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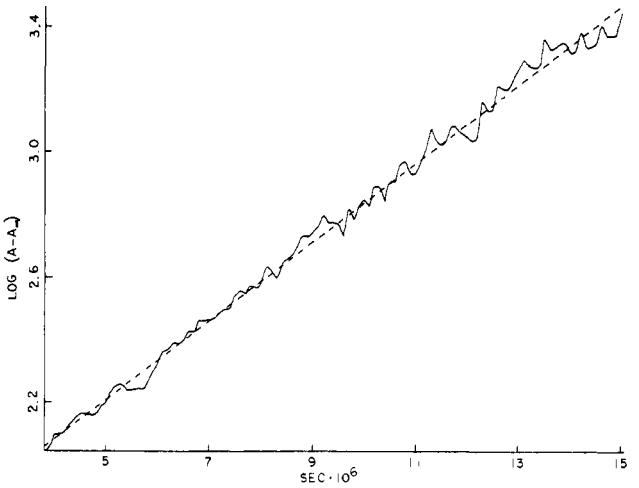


Figure 1. A plot of log $(A - A_\infty)$ vs. time for DHD + CO → DHD(CO) in benzene at [CO] = 2.30×10^{-4} M. The observed rate, the average of 16 voltage traces, is 1.25×10^5 s^{-1}.

Rougee and Brault in their equilibrium studies.[16] We have therefore undertaken the determination of all the rate constants in the interaction of heme with imidazole and CO in order to understand the effect of strain on the reactivity of the heme. We report kinetics of deuteroheme dimethyl ester with imidazole and 2-methylimidazole in benzene, and those of mesoheme dimethyl ester with 1-methylimidazole and 2-methylimidazole in aqueous cetyltrimethylammonium bromide (CTAB).

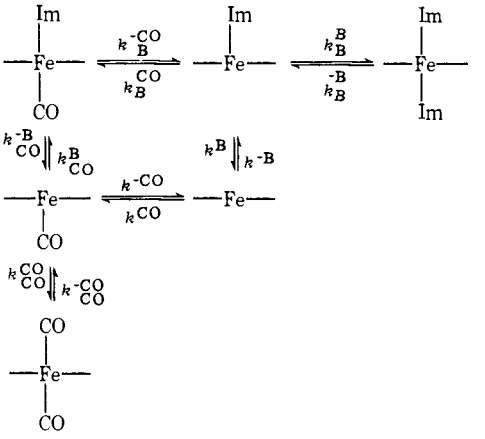
Experimental Section

Published procedures were used for the syntheses of deuteroporphyrin dimethyl ester (DHD)[19] and mesoporphyrin dimethyl ester (MHD)[20] and for iron insertion.[21] Imidazole (Im) and 2-methylimidazole (2-MeIm) were recrystallized twice before use; 1-methylimidazole (1-MeIm) was distilled. Carbon monoxide was from Matheson Gas Products, sodium dithionite from J. T. Baker, and cetyltrimethylammonium bromide (CTAB) from sigma. Visible spectra were recorded on a Cary 15 or Perkin-Elmer 550 spectrophotometer. The temperature in the kinetic runs was kept to 25.0 ± 0.1 °C with a circulating bath.

Benzene solutions were prepared with Mallinckrodt SpectrAR grade benzene. The hemin solution was reduced by the aqueous $Na_2S_2O_4$ technique described by Brault and Rougee.[22] The wet solution of heme was dried by bubbling with a stream of CO for 10 min. The CO was removed, when necessary, either by freeze–thaw cycles or by removing approximately one-third of the benzene under vacuum; the latter technique was generally more successful. The solutions were then made to the desired concentration of CO by addition of gaseous CO via syringe; a minimum of 5 min of vigorous shaking was necessary to ensure equilibrium. The solubility of CO in benzene at 25 °C was taken as 7.50×10^{-3} M, calculated from the average of the Bunsen coefficients of Horiuti (0.167) and Gjaldbaek (0.169) as reported by Gjaldbaek.[23a] These values are probably more accurate than those determined earlier.[23b-d] Rougee and Brault[16] took the solubility as 6.7×10^{-3} M; their equilibrium constants have been corrected to the higher CO solubility. Solutions of the imidazoles in benzene were deoxygenated either by three freeze–thaw cycles or by bubbling with benzene-saturated CO for 15 min.

Aqueous solutions contained 2% by weight CTAB (Sigma) in a pH 9.5 0.1 M potassium phosphate buffer. Samples were prepared in a "closed" cuvette[24] with a long, narrow neck, designed to accommodate an Applied Science Laboratories W-10 septum plug. The cuvette was filled with CTAB solution. A syringe needle was inserted through the plug, and the plug then inserted into the neck of the cuvette, the needle providing an exit for the excess aqueous CTAB. The heme was added as a solution in methanol via microliter syringe. It was reduced by the addition of 10 μL of saturated aqueous $Na_2S_2O_4$ (for 5-mL solution). Titrations were performed by additions of aqueous base or saturated aqueous CO (9.6×10^{-4} M at 25 °C[25]), taking care that the number of moles of added CO exceeded the total number of moles of heme in solution. In some cases this necessitated the use of large gas volume tonometers rather than closed cuvettes.

Scheme I



Kinetics Measurements. Kinetics measurements were performed by monitoring the transmittance change of a solution after a photolyzing light pulse. A Braun Model 2000 40 VCR flashgun was used for slow rates; the length of the pulse was determined by an adjustable iris and fiber optics light pipe (Edmund Scientific Co.) which controlled the amount of light reaching the flashgun photosensor. Typically, the pulse width was 150 μs with a decay rate after cutoff of 2×10^4 s^{-1}. Fast rates were measured with a Phase-R DL2100D tunable dye laser rated at 0.5 J/pulse. Rhodamine 6G (585 nm) in absolute ethanol was used as the dye. The pulse width was approximately 400 ns, and the maximum rate measurable ~1×10^6. Light from the flash was excluded from the photomultiplier by a Kodak 35 Wratten filter followed by a monochromator. The monitoring wavelength was selected by a Zeiss PMQ II monochromator, and the monitoring beam was produced by a 30-W tungsten lamp. The photolysis and monitoring beams entered the 1-cm cell at right angles. An RCA 1P21 photomultiplier detected transient changes in the solution following photolysis.

The light pulse, detected by a photodiode (Hewlett-Packard 5082-4203), was used to start data collection. The voltage trace was recorded by a CAMAC 6-bit 10-MHz analog-to-digital converter governed by a Zilog Z-80 microprocessor. Time frames from 0.1 μs to 1 s in 0.1-μs steps were available; the apparatus could be programmed to record a total of 256 points in up to 16 different sequential time frames. The apparatus recorded the first, last, and sum of all traces; between 5 and 25 voltage traces were added for each run. The averaged voltage excursion was converted to $A - A_\infty$, and plots of log $(A - A_\infty)$ vs. time for 2 half-lives gave pseudo-first-order rates (an example is shown in Figure 1). The standard deviation within a run was generally less than 1%. Each rate was determined on at least two separate samples. Where a rate is referred to as being dependent on the concentration of a ligand, at least four different concentrations of that ligand were measured, unless otherwise noted.[26]

Treatment of Experimental Data

A heme can bind either one or two axial ligands; for a base and carbon monoxide this results in three hexacoordinated species (HBB, $H(CO)_2$, and HBCO), two pentacoordinated species (HB and HCO), and the four-coordinated heme H, interrelated as shown in Scheme I. The rate constants in Scheme I refer to addition or loss of a ligand. The subscript gives the ligand remaining through the reaction; the superscript gives the ligand added (or lost). Thus k_B^{CO} is the rate constant for HB → HBCO and k_B^{-CO} the rate constant for HBCO → HB. The equilibrium constants are defined in the same manner.[27]

The experiments in benzene were run with deuteroheme dimethyl ester, in order to utilize the equilibrium constants measured by Rougee and Brault.[16] Those in CTAB were run with mesoheme dimethyl ester, in order to compare the rate constants for the external bases with those previously measured for the "chelated hemes".[15]

Equilibrium Measurements. The mathematical analysis of

the spectrophotometric titration of a heme by a ligand and CO has been presented in detail by Rougee and Brault,[16] and is only summarized here. For equilibria involving only a single ligand, B, there are two equilibrium constants:

$$H + B \overset{K^B}{\rightleftharpoons} HB + B \overset{K_B^B}{\rightleftharpoons} HB_2$$

When $K_B^B = 0$, a titration of the heme with ligand shows an isosbestic point, and K^B may be derived from the usual expression

$$\log \left| \frac{A_0 - A}{A - A_\infty} \right| = \log K^B + \log [B] \qquad (1)$$

where A_0, A, and A_∞ are the absorbances of the initial, intermediate, and final equilibrium mixtures, respectively. If the equilibrium constant is low, and thus an accurate determination of A_∞ impossible, the following expression can be used:

$$\frac{1}{A_0 - A} = \frac{1}{A_0 - A_\infty} + \frac{1}{A_0 - A_\infty} \frac{1}{K^B} \frac{1}{[B]} \qquad (2)$$

When $K^B \simeq K_B^B$, all three species are seen, and measurement of absorbance at a wavelength where ϵ_H and ϵ_{HBB} are equal gives [HB] as a function of [B]. It has been shown that the concentration of HB reaches a maximum $[HB]_{max}$ at some $[B]_{max}$ where[17]

$$A_{max} - A_0 = (\epsilon_{HB} - \epsilon_H)[HB] \qquad (3)$$

$$[B]_{max} = (K^B K_B^B)^{-1/2} \qquad (4)$$

$$[HB]_{max} = \frac{[H_0](K^B/K_B^B)^{1/2}}{2 + (K^B/K_B^B)^{1/2}} \qquad (5)$$

Equations 3-5 allow determination of K^B and K_B^B.

For titrations of a heme by a base in the presence of a constant CO concentration the absorbance is related to the apparent equilibrium constant:

$$\frac{A_0 - A}{A - A_\infty} = K_{app}^B [B] \qquad (6)$$

In cases where the heme is saturated with CO before the base is added, K_{app}^B is K_{CO}^B. More complicated expressions pertain at lower [CO]; the titrations reported herein are all described accurately by $K_{app}^B = K_{CO}^B$. A similar situation applies for the titration of the heme by CO in the presence of a large excess of base. The equilibrium constants of Scheme I are related:

$$K^B K_B^{CO} = K^{CO} K_{CO}^B \qquad (7)$$

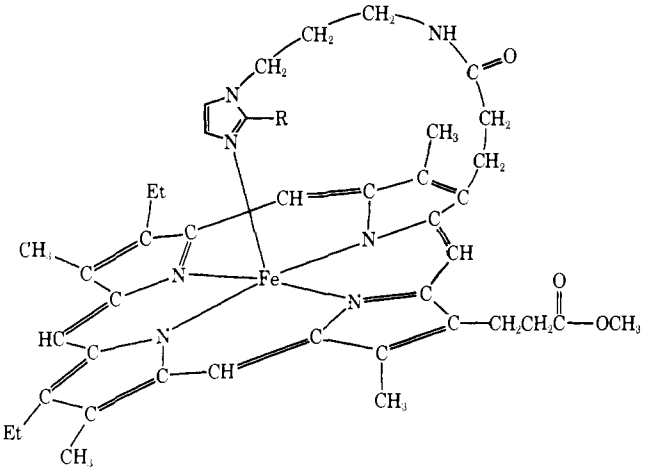
Kinetics Measurements. As seen in Scheme I, a full kinetic description of the heme-base-CO system requires 12 rate constants. In a mixture of heme, imidazole, and CO, the equilibria favor HBCO heavily, and the hexacoordinated heme is the dominant form except at very low [B] and [CO]. Flash photolysis of HBCO results in loss of CO to give HB, which returns to HBCO, but not necessarily by direct addition of CO. Analysis of the return to HBCO is complicated, but four limiting cases allow interpretation of the data.

(a) The return proceeds entirely through five-coordinated heme. This is the simplest of the approximations: that HB returns to HBCO only by direct addition of CO. Scheme I therefore simplifies to

$$HBCO \rightleftharpoons HB \rightleftharpoons HBB$$

and only four rates are of consequence. This approximation has been used in a number of studies, particularly in stopped-flow investigations of HBB against CO, as treated in more detail in the Discussion. The approximation was not used in this study because our reactions show some addition to four-coordinated heme under all conditions.

(b) The return proceeds entirely through four-coordinated heme. In this case the HB produced after the flash loses base to give the four-coordinated heme H which adds CO to give HCO and then base to give HBCO. This $HB \rightleftharpoons H \rightleftharpoons HCO \rightleftharpoons HBCO$ base elimination pathway has been shown to be important in the kinetics of chelated mesoheme **1a** at low pH[15a]



1a, R = H
b, R = CH_3

and of hindered chelated mesoheme **1b** at all pH values.[15b] In mixtures of heme with CO and external bases appropriate choices of [base] and [CO] create conditions where all of the reaction proceeds via the base-elimination pathway, and allow determination of the rates of loss of base from HB, k^{-B}, and the addition of B to HCO, k_{CO}^B.

(c) The return proceeds through four- and five-coordinated heme, and can be expressed as a base equilibrium followed by rate-limiting addition of CO. If the base on and off rates are fast with respect to the CO addition rates then the reactions involving base act as equilibria and the return to HBCO acts as an equilibrium ($HBB \rightleftharpoons HB \rightleftharpoons H$) followed by rate-determining addition of CO ($k_B^{CO}[CO] + k^{CO}[CO]$) to a second equilibrium ($HCO \rightleftharpoons HBCO$). The $HCO \rightleftharpoons HBCO$ equilibrium lies heavily on the side of the hexacoordinated HBCO, so the overall expression appears as the return from five-coordinated heme, k_B^{CO}, and the return from four-coordinated heme, k^{CO}, multiplied by the concentration of each of these species:

$$k_{obsd}/[CO] = k_B^{CO}[HB] + k^{CO}[H] \qquad (8)$$

This expression assumes that the CO additions are pseudo first order. Equation 8 may be rewritten, substituting expressions for [H] and [HB]:

$$[H] = \frac{[H]_0}{1 + K^B[B] + K^B K_B^B [B]^2} \qquad (9)$$

$$[HB] = \frac{[H]_0 K^B [B]}{1 + K^B[B] + K^B K_B^B [B]^2} \qquad (10)$$

$$\frac{k_{obsd}}{[CO]} = \frac{(k^{CO} + k_B^{CO} K^B [B])[H]_0}{1 + K^B[B] + K^B K_B^B [B]^2} \qquad (11)$$

A plot of the observed rate multiplied by $(1 + K^B[B] + K^B K_B^B [B]^2)$ vs. [B] gives a slope $k_B^{CO} K^B$ and an intercept k^{CO}. All of the experiments in this approach utilized [CO], [B] > $[H_0]$ such that the reactions were pseudo first order. Kinetic analyses used the standard $k_{obsd} = \log (A - A_\infty)$.

(d) In cases where no approximation is adequate, the kinetics have been simulated by computer fitting of Scheme I to the data. Two programs, an Euler algorithm and a modification of the DVOGER routine from the IMSL Scientific Library, have been used.[28] In both cases the rate of return to HBCO was calculated as a function of time. The known rate constants were entered, and the unknown rate constants (a maximum of 2)

Table I. Equilibrium Constants for Heme-Base-CO Mixtures

	Im, benzene[a] DHD[b]	2-MeIm, benzene[a] DHD[b]	1-MeIm, CTAB[c] MHD[d]	2-MeIm, CTAB[c] MHD[d]
K^B	4.5×10^3	1.3×10^4	34	85.5
K_B^B	6.8×10^4	-	33	-
K^{CO}	4.5×10^4	4.5×10^4	5×10^5	5×10^5
K_B^{CO}	4.3×10^8	2.2×10^6	7×10^8	4×10^6
K_{CO}^B	4.3×10^7	6.5×10^5	5×10^4	7×10^2

[a] The data in benzene are from ref 16, corrected for the revised solubility of CO in benzene (see Experimental Section). The Im equilibria are ±30%, the 2-MeIm ±20%, K^{CO} ±10%. [b] Deuteroheme dimethyl ester. [c] 2.0% cetyltrimethylammonium bromide in 0.1 M pH 9.5 phosphate buffer. [d] Mesoheme dimethyl ester.

were stepped by an order of magnitude in order to obtain the best fit to the experimental data. Thus rate constants determined by this procedure are accurate only to an order of magnitude. The modeling was used both to predict rate constants, in order to design experiments, and to calculate those rate constants not amenable to experimental measurement.

Results

Equilibria. The equilibria given in Table I for solutions of mesoheme dimethyl ester in aqueous CTAB were measured in this work. Rougee and Brault have measured the equilibria for deuteroheme dimethyl ester in benzene with CO, Im, and 2-MeIm; their equilibrium constants are also shown in Table I.[16]

Titration of the four-coordinated heme with 2-MeIm in CTAB buffer gave the five-coordinated HB with a well-defined isosbestic point. The equilibrium constant was too low to determine A_∞ accurately. Therefore, the value of $K^B = 85.5 \pm 1.0\ M^{-1}$ was obtained from a plot of $1/(A_0 - A)$ vs. $1/[2\text{-MeIm}]$ (eq 2).

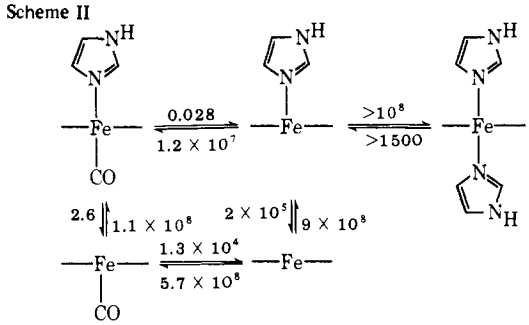
Titration of the four-coordinated heme by 1-MeIm was complicated by the simultaneous formation of HB and HBB. In such a situation the equilibrium constant can be obtained by measurement of the absorbance at a wavelength where ϵ_H and ϵ_{HBB} are equal, as discussed above. The titration was monitored at 421 nm, an isosbestic point for reduced MHD and MHD(1-MeIm)$_2$. Since the ϵ_{HB} of the monoimidazole heme is not known, that of the chelated mesoheme mono-3-(1-imidazoyl)propylamide monomethyl ester (**1a**) was used (ϵ 8.2×10^4 at 421 nm). The values of $K^B = 34 \pm 2$ and $K_B^B = 33 \pm 2\ M^{-1}$ were derived from eq 3-5.

Titration of mesoheme dimethyl ester with CO was apparently complicated by traces of binding impurities in the CTAB, which raised the apparent K^{CO}. Titration gave a series of spectra with a well-defined isosbestic point up to saturation, indicating the absence of H(CO)$_2$. However, the various runs showed some variation in K^{CO}, leading to a large standard deviation. Treatment of the data according to eq 1 gave $K^{CO} = (4.7 \pm 2) \times 10^5$, somewhat lower than previously reported.[15]

When MHD under an atmosphere of CO was titrated with 2-MeIm, the best runs were isosbestic at 406 nm with a slope of 1.00 ± 0.05 for the log $(A_0 - A)/(A - A_\infty)$ vs. log [2-MeIm] plots. Other runs were either not entirely isosbestic or showed some curvature in the log-log plot. The difficulty again seems to arise from impurities in the CTAB, which compete with the 2-MeIm in binding to the HCO. The value of K_{CO}^B thus has a relatively large standard deviation, $K_{CO}^B = (7 \pm 2) \times 10^2\ M^{-1}$. This value for K_{CO}^B, in conjunction with those for K^{CO} and K^B, gave a $K_B^{CO} = 4 \times 10^6\ M^{-1}$ (eq 7).

Titration of MHD with 1-MeIm in the presence of [CO] showed an isosbestic point at 406.5 nm. K_B^{CO} was calculated from eq 1 at 403 and 410 nm for samples with both 8.0×10^{-5} and 9.6×10^{-4} M CO; no effect of either wavelength or [CO] was seen. The value of $K_{CO}^B = (5.1 \pm 0.5) \times 10^4\ M^{-1}$, with those of K^{CO} and K^B, gave a value for K_B^{CO} of $7 \times 10^8\ M^{-1}$ (eq 7).

Scheme II



Kinetics

Carbon Monoxide in Benzene and in CTAB. The rate of addition of CO to deuteroheme dimethyl ester was measured in a two-phase system consisting of benzene and a saturated solution of $Na_2S_2O_4$ in pH 7.3 buffer. Even in water-saturated benzene (3×10^{-2} M H_2O[29]) the value of 10 for $K_{CO}^{H_2O}$[16] is sufficiently low that, at [CO] $\sim 10^{-4}$, HCO accounts for about 70% of the mixture. Under these conditions, photolysis produced the four-coordinated heme, which returned directly to HCO. The linear plot of the observed rate vs. [CO] gave a k^{CO} of $(5.7 \pm 0.3) \times 10^8\ M^{-1}\ s^{-1}$. This value for k^{CO}, in conjunction with the equilibrium constant, $K^{CO} = 4.5 \times 10^4\ M^{-1}$, gives a value for loss of CO $k^{-CO} = 1.3 \times 10^4\ s^{-1}$.[30]

The rate of addition of CO to mesoheme dimethyl ester in aqueous CTAB was also linearly dependent on [CO] with a rate, k^{CO}, of $(1.9 \pm 0.2) \times 10^8\ M^{-1}\ s^{-1}$. Since titration of the heme in CTAB with CO gave no evidence for the H(CO)$_2$ species, k_{CO}^{CO} is neglected in aqueous solution.

Carbon Monoxide and Imidazole in Benzene. The general outline of the heme-base-CO kinetics for imidazole in benzene is shown in Scheme II. Hexacoordinated HBCO is the only species in solution except at very low concentrations of imidazole and CO. Photolysis of HBCO gives the five-coordinated HB, which can disappear in three ways: it can add a second imidazole to give HBB, it can add a CO to give HBCO, or it can lose its imidazole to give the four-coordinated heme, H. The way in which it disappears is a function of the concentration of imidazole and carbon monoxide, and, by controlling these concentrations, it is possible to determine not only these three rates, but all of the rates shown in Scheme II.

(a) Low [Im] and Low [CO], Determination of k^{-B}. Even when the concentrations of imidazole and carbon monoxide are roughly equal to that of the heme, [Im] $\simeq$ [CO] $\simeq$ [heme] $\simeq 1 \times 10^{-5}$, the hexacoordinated HBCO is still the major species in solution. Flash photolysis of such a solution produced HB which disappeared with an initial rate of $2 \times 10^5\ s^{-1}$, a rate which must be addition of imidazole, loss of imidazole, or addition of CO to HB. The rate was independent of the concentration of imidazole in the range $1 \times 10^{-5} < [Im] < 5 \times 10^{-5}$, and thus did not correspond to addition of a second imidazole to HB. Addition of CO to HB would proceed with a rate

$k_B^{CO}[CO]$ or $\sim 10^2\ s^{-1}$ for $k_B^{CO} = 1.2 \times 10^7\ M^{-1}\ s^{-1}$ and [CO] $\simeq 10^{-5}$ M, far slower than the observed rate. Thus the observed rate is neither addition of imidazole, since the rate is independent of the concentration of imidazole, nor addition of CO, since CO addition is too slow, and must be loss of imidazole from HB.

Knowing k^{-B}, the rate of loss of imidazole from the five-coordinated heme, it is possible to calculate the rate of addition of imidazole to the four-coordinated heme, k^B, since the equilibrium constant, K^B, relating these species is known from work of Rougee and Brault: $k^B = k^{-B}K^B = 9 \times 10^8\ M^{-1}\ s^{-1}$.

(b) Low [Im] and High [CO], Determination of k_{CO}^B. Flash photolysis of a solution with a low concentration of imidazole ($1\text{-}5 \times 10^{-5}$ M) and an atmospheric concentration of CO (7.5×10^{-3} M) gives HB. At these concentrations the HB disappears both by addition of CO and by loss of imidazole, but not by addition of a second imidazole, since [Im] is too low to allow this pathway to compete, i.e., $k_B^{CO}[CO] \simeq k^{-B} \simeq 10^5 > k_B^B[B] = 10^3\text{-}10^4$. Any heme molecule which adds CO forms HBCO and is no longer subject to measurement. However, those molecules which lose base form the four-coordinated heme, H. This H can either add CO or add imidazole, but the former reaction is far faster, and thus a population of HCO builds rapidly. Because addition of CO to the four-coordinated heme, H, is faster than loss of imidazole from HB to form H ($k^{CO}[CO] \simeq 4 \times 10^6 > k^{-B} = 2 \times 10^5$), the reaction appears as a rapid formation of HCO. This HCO then adds imidazole to re-form the starting HBCO. Kinetically, the overall reaction appears as a fast initial rate, independent of [Im], corresponding to a mixture of $k_B^{CO}[CO]$ and k^{-B}, and a subsequent slower rate, dependent on [Im], corresponding to $k_{CO}^B[Im]$. A plot of the slower base-dependent rate against [Im] gave a slope of $(1.1 \pm 0.1) \times 10^8\ M^{-1}\ s^{-1}$ corresponding to k_{CO}^B.

Since the equilibrium constant between HCO and HBCO is known for imidazole in benzene,[16] $K_{CO}^B = 4.3 \times 10^7\ M^{-1}$, the rate of loss of imidazole from the hexacoordinated HBCO can be calculated: $k_{CO}^{-B} = k_{CO}^B/K_{CO}^B = 2.6\ s^{-1}$. This value is in good agreement with preliminary estimates of $2\text{-}10\ s^{-1}$ determined by NMR saturation transfer experiments for a mixture of protoheme dimethyl ester, CO, and 1-methylimidazole.[31]

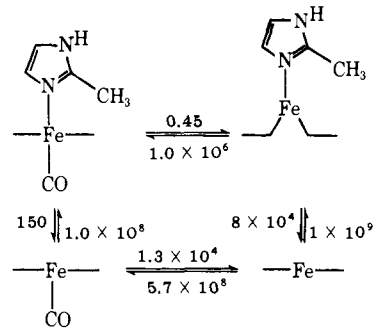
The above discussion ignores the presence of any $H(CO)_2$. If the HCO $\rightleftharpoons$ $H(CO)_2$ equilibrium were established before HCO added imidazole, the observed rate k_{CO}^B would be about half that of the true rate, since about 50% of the heme would be tied up as the hexacoordinated $H(CO)_2$ ($K_{CO}^{CO} = 190$,[16,32] $[CO] = 7.5 \times 10^{-3}$ M). Experiments with 2-MeIm and CO, discussed below, indicate that little of the heme forms $H(CO)_2$. Therefore the observed rate constant assigned to return from HCO to HBCO is close to the true rate constant for this step.

(c) High [Im] and Low [CO], Determination of k^{CO} and k_B^{CO}. Under conditions of low [CO] $\simeq 5 \times 10^{-5}$ M and high [Im] $= 1\text{-}8 \times 10^{-3}$ M photolysis of HBCO produces HB. Addition of CO to HB ($k_B^{CO}[CO] \simeq 6 \times 10^2\ s^{-1}$) is slow compared to either loss of imidazole from HB ($k^{-B} = 2 \times 10^5\ s^{-1}$) or addition of imidazole to HB ($k_B^B[B] > 10^5\ s^{-1}$). The rate appears as a preequilibrium (HBB $\rightleftharpoons$ HB $\rightleftharpoons$ H) followed by rate-limiting addition of CO. This is expressed by eq 11, and a plot of $k_{obsd}(1 + K^B[Im] + K^BK_B^B[Im]^2)$ vs. [Im] according to eq 11 gave a slope of $k_B^{CO}K^B$, for a k_B^{CO} of $(1.2 \pm 0.3) \times 10^7\ M^{-1}\ s^{-1}$.

This value for the addition of CO to HB, in conjunction with that for the equilibrium between HB and HBCO ($K_B^{CO} = 4.3 \times 10^8$) allows calculation of the rate of loss of CO from HBCO: $k_B^{-CO} = k_B^{CO}/K_B^{CO} = 0.028\ s^{-1}$.

(d) High [Im] and High [CO], Determination of k_B^B. When the concentrations of both imidazole and carbon monoxide are high, the HB produced after the flash disappears by all three pathways. The complexity of this situation precludes any simple analysis of the rate of appearance of HBCO, but limits on k_B^B can be set. At atmospheric CO (7.5×10^{-3} M), varying the imidazole concentration from 10^{-3} to 9×10^{-3} M caused the observed first-order decay to change from 1300 to $160\ s^{-1}$. If none of the HB were adding imidazole to give the hexacoordinated HBB, the rate would be independent of [Im] and fast ($k_B^{CO}[CO] = 9 \times 10^4\ s^{-1}$). Since neither of these is true, addition of imidazole must compete with addition of CO, or $k_B^B[Im] \geq k_B^{CO}[CO]$. This sets a lower limit of $\sim 10^8$ for k_B^B. A more thorough analysis, using a computer simulation of the rate of appearance of HBCO, indicated a value of k_B^B of $\sim 10^9\ M^{-1}\ s^{-1}$.

Scheme III



Carbon Monoxide and 2-Methylimidazole in Benzene. Analysis of the heme-base-CO kinetics of 2-MeIm is somewhat easier than analysis of the heme-base-CO kinetics of Im itself, since 2-MeIm does not bind twice to the heme. This lack of formation of HBB with 2-MeIm is due to the steric interference of the imidazole methyl group with the porphyrin ring. In Scheme III, therefore, only one hexacoordinated (HBCO), two pentacoordinated (HB, HCO), and one four-coordinated (H) species are kinetically important.

(a) Low [2-MeIm] and High [CO], Determination of k^{-B} and k_{CO}^B. For 2-MeIm, flash photolysis of HBCO produces HB which has only two avenues open to it: it can add CO or lose 2-MeIm. Photolysis of a solution with low [2-MeIm], $1\text{-}6 \times 10^{-5}$ M, and atmospheric CO gave a solution of HB which disappeared at $(8 \pm 1) \times 10^4\ s^{-1}$. Since the rate of CO addition under these conditions, $k_B^{CO}[CO] = 7.5 \times 10^3\ s^{-1}$, is too slow to account for the reaction, the observed rate must correspond to loss of base, k^{-B}. As expected the observed k^{-B} was independent of the concentration of 2-MeIm. Loss of base is followed by a very fast addition of CO to give HCO, $k^{CO}[CO] = 4.3 \times 10^6\ s^{-1}$. The HCO then adds 2-MeIm to form HBCO. Since the addition of CO is too fast to measure, the overall reaction after the flash is biphasic, corresponding to a fast loss of base from HB (k^{-B}), followed by a slower addition of base to HCO (k_{CO}^B). This slower rate was linearly dependent on the concentration of 2-MeIm for [2-MeIm] $= 2\text{-}6 \times 10^{-5}$ M. A plot of this second, slower rate against [2-MeIm] gave a straight line with a slope, k_{CO}^B, of $(1.0 \pm 0.1) \times 10^8\ M^{-1}\ s^{-1}$ (Figure 2).

In order to verify these assignments, and to investigate the role of the hexacoordinated $H(CO)_2$ in the kinetic scheme, a spectral intermediate study was performed. Photolysis of a solution containing [2-MeIm] $= 4.2 \times 10^{-5}$ and [CO] $= 7.5 \times 10^{-3}$ M was observed at 11 wavelengths at intervals after the flash (Figure 3). The first spectrum after the flash, at 5 μs, corresponds to HB. The HB loses 2-MeIm at $8 \times 10^4\ s^{-1}$ (k^{-B}) to give a new spectrum which reaches a maximum at 35 μs. This spectrum shows minima at 390 and 425 nm and a maximum at 407 nm. These correspond to those of the difference spectrum between a mixture of HCO and $H(CO)_2$ (atmospheric CO) and HBCO (Figure 3B). The peak at 407 nm is

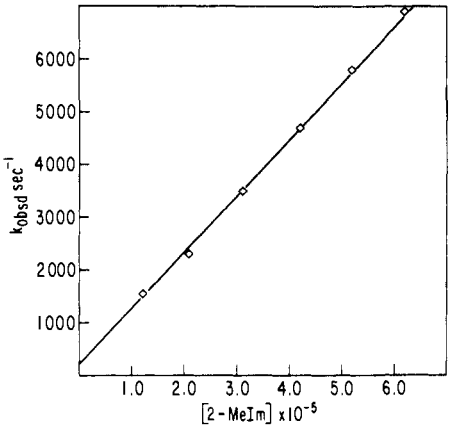


Figure 2. The observed rate of addition of 2-MeIm to DHD(CO) at [CO] = 7.5 × 10^{-3} M and varying [2-MeIm]. The slope is k^{B}_{CO}.

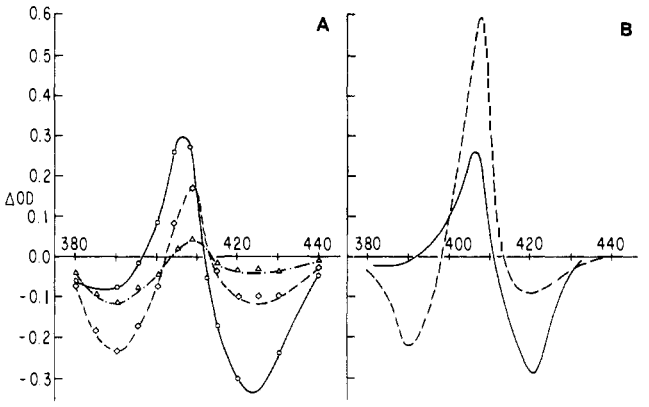
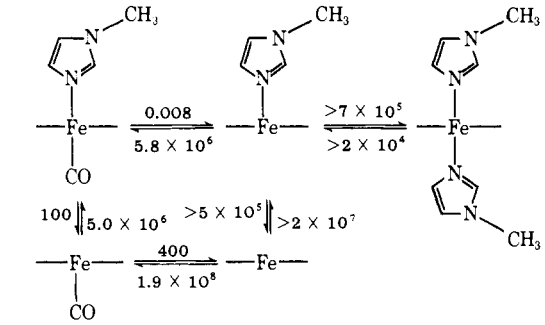


Figure 3. (A) A plot of the difference spectra for a solution of deuteroheme dimethyl ester in benzene with [CO] = 7.5 × 10^{-3} and [2-MeIm] = 4.2 × 10^{-5} M at intervals after the flash. The points are experimental, and the lines are smooth curves drawn through the points: (○) 5 μs; (◇) 35 μs; (△) 140 μs after the flash. (B) Difference spectra, deuteroheme dimethyl ester in benzene at atmospheric [CO]: (—) H(CO)(2-MeIm) − H(2-MeIm); (---) H(CO)(2-MeIm) − [H(CO) + $H(CO)_2$].

much smaller in the kinetic difference spectrum than in the static difference spectrum, indicating that only a small amount of the heme has added a second CO to give $H(CO)_2$. The HCO then adds 2-MeIm to form HBCO. This is seen in Figure 3A, where the spectrum at 35 μs returns isosbestically to the base line (for clarity, only one spectrum, that at 140 μs, is shown). The spectral intermediate thus verifies that the reaction appears as loss of base from HB, followed by addition of base to HCO. In addition, it indicates that $H(CO)_2$ is contributing very little to the overall reaction. If the HCO ⇌ $H(CO)_2$ equilibrium were fully established before reaction of HCO with 2-MeIm, the observed rate would be the true rate multiplied by the fraction of the heme found as HCO or $k_{obsd} = k^{B}_{CO}[2\text{-MeIm}](1 + K^{CO}_{CO}[CO])$, for a k^{B}_{CO} of 2.2 × 10^{8} M^{-1} s^{-1}. The difference spectrum shows little $H(CO)_2$, and therefore the value of k^{B}_{CO} is probably closer to $k_{obsd}/[2\text{-MeIm}]$ or 1 × 10^{8} M^{-1} s^{-1}.

(b) High [2-MeIm] and Low [CO], Determination of k^{CO} and k^{CO}_{B}. Photolysis of a solution with a high [2-MeIm] and a low [CO] gives HB which loses 2-MeIm to give the four-coordinated heme, H. This four-coordinated heme can either readd 2-MeIm or add CO. For [2-MeIm] between 3 × 10^{-4} and 10^{-3} and [CO] ≃ 1 × 10^{-4} M, addition of 2-MeIm predominates (k^{B}[B] = 3 × 10^{5} to 10^{6}, k^{CO}[CO] ≃ 6 × 10^{4} s^{-1}). Therefore, addition of CO to the heme appears as a preequilibrium (H ⇌ HB) followed by rate-limiting addition of CO. Flash photolysis gave a return to HBCO that was close to linear in [CO] and which decreased with increasing [2-MeIm], as predicted. Each k_{obsd} was first order, within experimental error, but the rates were wavelength dependent, decreasing by almost a factor of 2 from 404 to 412 nm. This wavelength dependence arises because the addition of CO is not cleanly rate determining. The problem is due to constraints on the CO and 2-MeIm concentrations. First, it is necessary to have [CO] at least 5 × 10^{-5} M in order to have pseudo-first-order kinetics in the addition of CO to the heme ([heme] ≃ 5 × 10^{-6} M). This concentration of CO gives an observed rate for CO addition to four-coordinated heme, k^{CO}[CO], of 3 × 10^{4} s^{-1}. Second, it is necessary to have at least a fivefold change in the concentration of 2-MeIm in order to test the dependence of k_{obsd} on [2-MeIm] adequately. The solubility of 2-MeIm in benzene is reported as 3 × 10^{-2} M[16] and in our hands even solutions of 1 × 10^{-2} M crystallize upon standing. We have used concentrations of 2-MeIm from 1 × 10^{-3} to 1 × 10^{-2} M in this experiment. These concentrations of 2-MeIm give values for addition of 2-MeIm to HCO, k^{B}_{CO}[B], of 10^{5}-10^{6} s^{-1}. Thus, at the lower concentration of base used, the rate of base addition to HCO is too close to the rate of CO addition to H for the latter to be cleanly rate limiting. This similarity in rates would not be a problem if the reaction were going primarily by simple

Scheme IV



readdition of CO to HB. However, even though substantial amounts of HB are present (93-99% of the mixture), the rate of addition of CO to HB is so much slower than that of addition to H that the majority of the reaction (98-81%) proceeds via the base-elimination mechanism. These considerations place some strain on the interpretation of the data obtained from this experiment. Nonetheless, the k_{obsd} were close to first order, and the change in rate as a function of [2-MeIm] did not appear wavelength dependent. A plot of k_{obsd} vs. $[2\text{-MeIm}]^{-1}$ according to a modified eq 11 ($K^{B}_{B} = 0$, $K^{B} \gg 1$) (Figure 4)

$$k_{obsd} = k^{CO}/K^{B}[2\text{-MeIm}] + k^{CO}_{B} \qquad (12)$$

gave a k^{CO}_{B} (intercept) of (1.0 ± 0.3) × 10^{6} M^{-1} s^{-1} and a k^{CO}, derived from the slope, of (4.7 ± 1.5) × 10^{8} M^{-1} s^{-1}, this last within experimental error of the value k^{CO} = (5.7 ± 0.3) × 10^{8} M^{-1} s^{-1} determined without base present.

Carbon Monoxide and 1-Methylimidazole in Aqueous CTAB. Kinetic Scheme IV for 1-MeIm, CO, and mesoheme dimethyl ester in aqueous CTAB is similar in most respects to that of Im, CO, and deuteroheme dimethyl ester in benzene. Again, the HB produced by photolysis of HBCO can react in three ways: by addition of 1-MeIm, addition of CO, or loss of 1-MeIm.

(a) Low [1-MeIm] and Low [CO], Determination of k^{-B}, k^{B}_{CO}, and k^{-B}_{CO}. Even at low concentrations of both [1-MeIm] and [CO] (~5 × 10^{-5} M), HBCO is still the major heme species in solution. Photolysis of such a solution produced HB. The course of the reaction was followed at five wavelengths, and in each case only direct production of HCO, with a rate of k^{CO}[CO], was seen. The four-coordinated heme was not detected as an intermediate, and thus its formation was too fast to measure with the present apparatus, or $k^{-B} \geq 5 \times 10^{5}$ s^{-1}. The formation of HCO was followed by a slower addition of

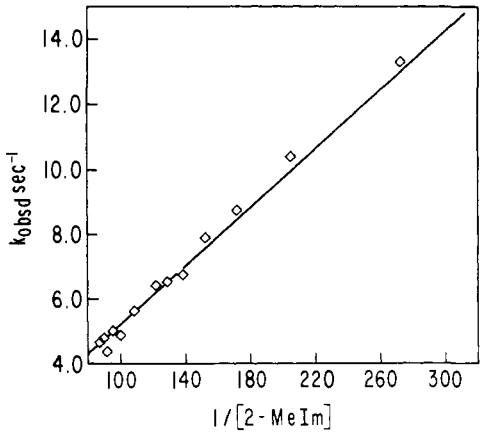


Figure 4. A plot of the observed rate of return to DHD(2-MeIm)(CO) as a function of 1/[2-MeIm] in benzene; [CO] = 1.2×10^{-4} M.

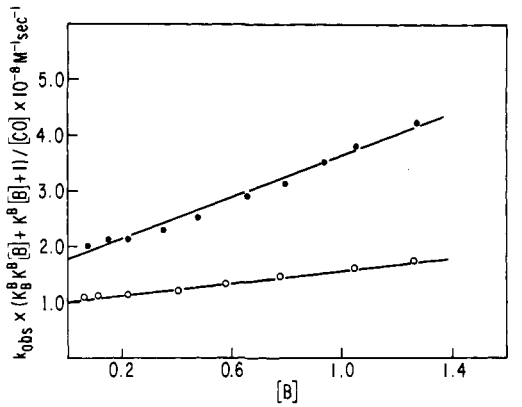


Figure 5. A plot of $k_{obsd}(1 + K^B[B] + K_B^B K^B[B]^2)/[CO]$ vs. [B] in pH 9.5, 2% CTAB: (●) 1-MeIm, [CO] = 4.0×10^{-4} M; (○) 2-MeIm, [CO] = 4.0×10^{-4} M.

1-MeIm to form HBCO. This return to HBCO was linearly dependent on [1-MeIm] and independent of [CO]. A plot of k_{obsd} vs. [1-MeIm] gave a slope k_{CO}^B of $(5.0 \pm 0.4) \times 10^6$ M^{-1} s^{-1}.

This value for k_{CO}^B, in conjunction with the equilibrium constant between HCO and HBCO, $K_{CO}^B = 5 \times 10^4$ M^{-1}, allows determination of the rate of loss of 1-MeIm from the hexacoordinated HBCO: $k_{CO}^{-B} = k_{CO}^B/K_{CO}^B = 100$ s^{-1}.

(b) High [1-MeIm] and Low [CO], Determination of k_B^{CO} and k_B^{-CO}. Under conditions of high [1-MeIm] = 0.7-1.7 M and low [CO] = 4×10^{-5} M photolysis of HBCO produces HB. As discussed for Im in benzene, addition of CO (k_B^{CO}[CO] $\simeq 2 \times 10^2$ s^{-1}) is slow compared to either loss of base from HB ($k^{-B} > 5 \times 10^5$ s^{-1}) or addition of base to HB (k_B^B[1-MeIm] $> 10^5$; see below). Thus the overall reaction may be viewed as an equilibrium (H ⇌ HB ⇌ HBB) followed by a rate ($k^{CO} + k_B^{CO}K^B$[1-MeIm]), as expressed by eq 11. When k_{obsd} ($1 + K^B$[1-MeIm] $+ K^B K_B^B$[1-MeIm]^2) was plotted against [Im] the resulting slope gave $k_B^{CO}K^B$ for a k_B^{CO} of $(5.8 \pm 0.1) \times 10^6$ M^{-1} s^{-1}; the intercept, k^{CO}, was $(2.2 \pm 0.4) \times 10^8$ M^{-1} s^{-1} (Figure 5). This last is in good agreement with the value of k^{CO} determined with no base present, $k^{CO} = (1.9 \pm 0.2) \times 10^8$ M^{-1} s^{-1}.

Since the equilibrium constant between HB and HBCO has been calculated from our titrations, $K_B^{CO} = 7 \times 10^8$ M^{-1}, the rate of loss of CO from HBCO can be calculated: $k_B^{-CO} = k_B^{CO}/K_B^{CO} = 0.008$ s^{-1}. This value is close to that obtained in this solvent for chelated mesoheme **1a**, 0.015 s^{-1}.[33]

Initially, it was expected that the rate of addition of 1-MeIm to HB would be fast because all other base-addition rates were quite fast, including that of Im to HB in benzene. This expectation was borne out by the excellent fit of the experimental data to eq 11 for solutions with high [1-MeIm] and low [CO]. It was confirmed by recording the difference spectrum of the first intermediate seen after the flash in a solution with [1-MeIm] = 0.11 M and [CO] = 2×10^{-6} M. The kinetic difference spectrum shown in Figure 6 is that of HBB, as seen by comparison with the static difference spectrum. The kinetic difference spectrum was obtained with the flashgun; however, the laser revealed no other species, and therefore, under the conditions of the experiment, the dibase hexacoordinated HBB was formed with a rate $>10^5$ s^{-1}. Since establishment of an equilibrium is given by the sum of the forward and reverse reactions, k_B^B[B] $+ k_B^{-B} > 10^5$, or, substituting, $k_B^{-B}(1 + K_B^B$[1-MeIm]$) > 10^5$. Solving this expression gives $k_B^{-B} > 2 \times 10^4$ s^{-1} and $k_B^B > 7 \times 10^5$ M^{-1} s^{-1}.

Carbon Monoxide and 2-Methylimidazole in Aqueous CTAB. The kinetic scheme of 2-MeIm, mesoheme dimethyl ester, and CO in aqueous CTAB (Scheme V) is similar to that of 2-MeIm, deuteroheme dimethyl ester, and CO in benzene.

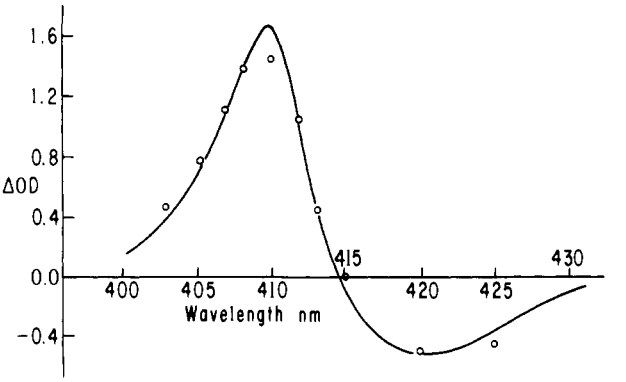


Figure 6. Difference spectra of MHD(1m)$_2$ and MHD(1m)(CO) in 2% pH 9.5 aqueous CTAB, [1-MeIm] = 0.108 M, [CO] = 2.15×10^{-6} M: (—) static difference spectrum; (○) ΔOD measured at 2.2 ms after the beginning of the flash. The flash length was 150 μs in this experiment; the observed rate of return to MHD(1m)(CO) was ~50 s^{-1}. The point at 410 nm appears low because the spectral bandwidth in the kinetics run was larger than that of the static spectrum in this experiment.

Again, the 2-MeIm does not bind twice to the heme. In addition, H(CO)$_2$ does not form in aqueous solution, and thus only HBCO, HB, H, and HCO need be considered. Problems arise in this system because the overall formation constant for HBCO from H is low ($\sim 3 \times 10^8$ M^{-2}) compared to the other systems studied ($\gtrsim 10^{10}$ M^{-2}). Thus reactions at low [2-MeIm] and low [CO] cannot be run as usual, since HBCO is not the major species in solution under these conditions.

(a) Moderate [2-MeIm] and Low [CO], Determination of k^{-B}. When [2-MeIm] = 10^{-3} M and [CO] = 5×10^{-5} M, HBCO is the dominant form in solution. Photolysis produces HB, and it was expected that loss of base k^{-B} would be faster than addition of CO to HB (k_B^{CO}[CO] = 25 s^{-1}). This proved to be the case; HB went directly to HCO, without the appearance of H

Scheme V

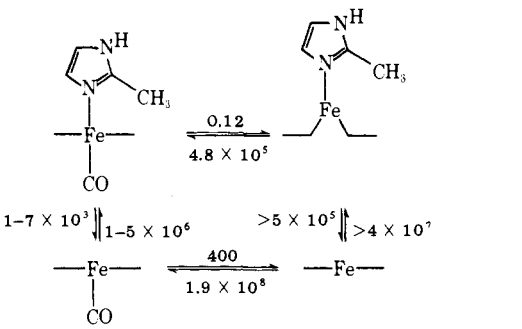


Table II. Geometry of Heme Complexes

complex	metal–porphyrin plane	N_ϵ–porphyrin plane	metal–N_ϵ	ref
deoxy-Hb α	0.60	2.6	2.0	35b
β	0.63	2.8	2.2	35b
deoxy-Mb	0.55	2.6	2.1	35c
2-MeImFe^{II}-TPP	0.55	2.68	2.16	35a
1-MeImCo^{II}-TPP	0.14	2.30	2.16	36
1,2-diMeIm-Co^{II}-TPP	0.18	2.37	2.22	37

as an intermediate. This implies that k^{-B} is faster than the limits of the apparatus or $k^{-B} > 5 \times 10^5\ s^{-1}$.

(b) High [2-MeIm] and Low [CO], Determination of k_{CO}^{B} and k_{CO}^{-B}. Under conditions of high [2-MeIm] and low [CO], photolysis produced HB which quickly established the HB $\rightleftharpoons$ H equilibrium. These species then added CO more slowly. The rate was dependent on [2-MeIm], increasing [2-MeIm] decreasing the amount of free four-coordinated heme and thus decreasing the rate. A plot of k_{obsd} against [2-MeIm] according to eq 11 gave a k_B^{CO}(derived from the slope) of $(4.8 \pm 1.4) \times 10^5\ M^{-1}\ s^{-1}$ and a k^{CO} (intercept) of $(1.4 \pm 0.3) \times 10^8\ M^{-1}\ s^{-1}$, this last in good agreement with the value derived from mesoheme dimethyl ester and CO in aqueous CTAB without ligand, $(1.9 \pm 0.2) \times 10^8\ M^{-1}\ s^{-1}$.

The value for k_B^{CO}, in combination with that of the equilibrium between HB and HBCO, $K_B^{CO} = 4 \times 10^6\ M^{-1}$, gives a rate for loss of CO from the hexacoordinated HBCO: $k_B^{-CO} = k_B^{CO}/K_B^{CO} = 0.12\ s^{-1}$.

(c) Moderate [2-MeIm] and High [CO], Determination of k_{CO}^{B} and k_{CO}^{-B}. The determination of k_{CO}^{B} is complicated by the difficulty in isolating this rate from the others in the scheme.

A clean determination of k_{CO}^{B} is possible only when the rate of 2-MeIm addition to HCO is slow compared to the rate of formation of HCO and fast compared to the loss of 2-MeIm from HBCO, e.g. (for a factor of 10 difference in the rates), when $10k_{CO}^{B}[2\text{-MeIm}] \leq k^{CO}[CO]$ and $k_{CO}^{B}[2\text{-MeIm}] \geq 10k_{CO}^{-B}$. These conditions allow only a narrow range of values for [2-MeIm]. It was possible, however, to estimate k_{CO}^{B} in this system by utilizing a sample with atmospheric [CO] and $2 \times 10^{-3} < [2\text{-MeIm}] < 10^{-2}$. The flash produced HB, which went quickly to HCO, and then more slowly to HBCO. The rates, as expected, were slightly curved and slightly wavelength dependent, but indicated a value of k_{CO}^{B} of $\sim 1 \times 10^6$. The true value of k_{CO}^{B} will be somewhat higher, since $k^{CO}[CO]$ and k_{CO}^{-B} both reduce the apparent value of k_{CO}^{B}. This range of values for k_{CO}^{B}, together with that calculated for the equilibrium between HCO and HBCO, $K_{CO}^{B} = 7 \times 10^2\ M^{-1}$, gives a range for the rate of loss of B from HBCO, $k_{CO}^{-B} = k_{CO}^{B}/K_{CO}^{B} = 1\text{–}7 \times 10^3$.

Discussion

The results above indicate that the rates of ligand association and dissociation are influenced both by the ligand itself and by the solvent. These rates may be discussed in connection with the detailed geometry of the heme, the ligand association and dissociation rates of heme proteins, and the general mechanistic considerations of heme ligation.

Geometry of the Heme. The stereochemistry of metalloporphyrins has been studied extensively in recent years.[34] The out of plane displacement of the metal is a function both of the size and spin of the metal and of the ligands on the axial positions. Ferrous porphyrins, with six d electrons, are generally in one of three spin states: high spin, five coordinated, with the iron atom displaced toward the single axial ligand; low spin, six coordinated, with the iron in the heme plane; and an intermediate species ($S = 1$) four-coordinated state in which the porphyrin displays a ruffled geometry. Deoxyhemoglobin and myoglobin are five-coordinated high-spin species exhibiting a substantial iron displacement (Table II), and under the Perutz hypothesis of cooperativity it is the amount of this displacement which primarily determines the reactivity of the heme.

We have used 2-MeIm and 1-MeIm (Im) to model the T and R state, respectively, of hemoglobin. The only crystal structure of a high-spin Fe(II) species presently available is that of FeTPP(2-MeIm).[35a] FeTPP(2-MeIm) is significantly domed, that is, the mean plane of the four porphyrin nitrogens is displaced toward the 2-MeIm axial ligand by 0.13 Å from the mean porphyrin core. Some of the doming may be a reflection of crystal packing constraints, but the structure does show significantly more doming than five-coordinated metalloporphyrins in general, where this distance is less than 0.05 Å.[34b] This doming arises from the steric interaction of the 2-methyl group with the plane of the porphyrin ring. The steric interaction serves to explain not only the doming, but also the fact that 2-MeIm does not bind twice to the heme either in aqueous solution[38a] or in benzene.

It would be desirable to compare the kinetic results for 1-MeIm (Im) with the crystal structure of a model for R-state deoxyHb. However, although the HB species is available kinetically by flash photolysis of HBCO, preparation of an unstrained five-coordinated ferrous porphyrin has been thwarted by the fact that addition of a second base has a higher equilibrium constant than addition of the first ($K_B^B > K^B$) causing the six-coordinated HBB to be the dominant form in solution. This implies that heme(1-MeIm) is less domed than heme(2-MeIm), although the precise magnitude of the effect is not known.

Cobalt porphyrins and hemoglobins have also been used to estimate the effect of doming on reactivity. In the cobalt systems $K^B > K_B^B$,[38b] allowing stable five-coordinated HB species to be prepared even for bases with no steric hindrance. Scheidt and co-workers have determined the crystal structures of 1-methylimidazolecobalt(II) tetraphenylporphyrin[36] and 1,2-dimethylimidazolecobalt(II) tetraphenylporphyrin[37] (Table II). For Co^{II}TPP, addition of a 2-methyl group to the axial imidazole induces a stretch of 0.07 Å in the N_ϵ–P_c distance, mostly in the Co–N_ϵ bond. This indicates that the 1-MeIm species is less domed than the 1,2-diMeIm species. Although the difference is small, the effect of the difference on O_2 affinities is large. The $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ of O_2 binding to 1-MeIm and 1,2-diMeIm complexes of substituted cobalt tetraphenyl porphyrins have been found to match those of R- and T-state cobalt hemoglobin, respectively.[13] The data on the cobalto-hemoglobin and the models, in conjunction with the X-ray parameters, imply that a small change in heme geometry is enough to account for the observed changes in oxygen equilibria. Our work establishes that this doming is consistent not only with the equilibria, but also with the kinetics of CO (and O_2[15]) binding to iron in hemoglobin.

Rates and Equilibria. Table III summarizes the rates measured in this work, and Table IV indicates the effect of steric constraint on these rates. We have used deuteroheme dimethyl ester in benzene and mesoheme dimethyl ester in CTAB in order to compare these results with others.[15,39] The kinetic difference between the two hemes is expected to be small. Sono et al. have reported that mesoheme adds CO about 40% faster than does deuteroheme in ethylene glycol.[39] Preliminary results from our laboratory indicate that mesoheme adds CO more slowly than does deuteroheme in benzene, by at most a factor of 2. Chelated hemes, however, do not show this difference; the CO addition rates, k_B^{CO}, for meso-, proto- and diacetyldeuterochelated hemes are all within experimental error of one another.[31] These small differences will affect the ratios re-

Table III. Rate Constants for Heme-Base-CO Mixtures[a]

rate	Im, benzene DHD	2-MeIm, benzene DHD	1-MeIm, CTAB MHD	2-MeIm, CTAB MHD
k^B, M^{-1} s^{-1}	9×10^8	1×10^9	$>2 \times 10^7$	$>4 \times 10^7$
k^B_{CO}, M^{-1} s^{-1}	1.1×10^8	1.0×10^8	5.0×10^6	$1\text{-}5 \times 10^6$
k^{CO}_B, M^{-1} s^{-1}	1.2×10^7	1.0×10^6	5.8×10^6	4.8×10^5
k^{-B}, s^{-1}	2×10^5	8×10^4	$>5 \times 10^5$	$>5 \times 10^5$
k^{-B}_{CO}, s^{-1}	2.6	150	100	$1\text{-}7 \times 10^3$
k^{-CO}_B, s^{-1}	0.028	0.45	0.008	0.12

[a] Abbreviations as in Table I. The values for k^B_{CO} and k^{-B} were measured directly; all other rate constants are derived either from the reverse rate and the equilibrium constant or from extrapolation of the observed rate to infinite concentration of base. Errors are generally ±10% (see text).

ported below but will not alter the conclusions reached. As expected, the hexacoordinated HBCO species is the most sensitive to steric strain because HBCO is planar and unable to accommodate the 2-methyl group of 2-MeIm easily. This strain is reflected most strongly in a comparison of the rate constants for the loss of unhindered and hindered imidazole from HBCO: $k^{-Im}_{CO}/k^{-2\text{-MeIm}}_{CO} = 0.017$ in benzene and 0.01–0.07 in CTAB. However, both loss of CO from HBCO (k^{-CO}_B) and addition of CO to HB (k^{CO}_B) show a substantial effect: $k^{-CO}_{Im}/k^{-CO}_{2\text{-MeIm}}$(benzene) = $k^{-CO}_{1\text{-MeIm}}/k^{-CO}_{2\text{-MeIm}}$(CTAB) = 0.06–0.07 and $k^{CO}_{Im}/k^{CO}_{2\text{-MeIm}}$(benzene) = $k^{CO}_{1\text{-MeIm}}/k^{CO}_{2\text{-MeIm}}$(CTAB) = 12. Interestingly, the rate of addition of imidazole to HCO, k^B_{CO}, is almost unaffected by the addition of a 2-methyl group. Apparently the transition state for adding a hindered ligand to HCO experiences little of the strain found in the product HBCO. These rates result in a substantial effect of steric hindrance on the equilibrium constants K^{CO}_B and K^B_{CO}. The large difference between K^{CO}_B for imidazole and 2-methylimidazole, $K^{CO}_{Im}/K^{CO}_{2\text{-MeIm}} \simeq 200$, derives from the increased strain of the H(2-MeIm)CO complex. A similar but somewhat smaller effect was observed for addition of imidazoles to HCO, $K^{Im}_{CO}/K^{2\text{-MeIm}}_{CO} \simeq 70$, due almost entirely to the base off rate k^{-B}_{CO}.

The five-coordinated HB is not sensitive to steric strain; the rate of addition of imidazole to H is almost unaffected by the presence of a 2-methyl group (Table IV), and the rate of loss of imidazole from HB is in fact faster for imidazole itself than for 2-MeIm. These are seen in a higher equilibrium constant for binding of 2-MeIm ($K^B = 1.3 \times 10^4$) than for binding Im ($K^B = 4.5 \times 10^3$), a reflection of the increased electron-donating property of 2-MeIm.

The rates of imidazole addition to HB, k^B_B, and loss from HBB, k^{-B}_B, have been estimated by the observation that the base addition and loss reactions appear fast with respect to addition of CO at high [Im] and low [CO]. These estimates have been corroborated by a study of Momenteau and Lavalette[40] which appeared after this work was completed. They used flash photolysis to measure directly a k^B_B of 1.4×10^8 M^{-1} s^{-1} for $TPP(Im)_2$ in toluene at 25 °C. These rates ($k^B_B > 10^8$, $k^{-B}_B > 1500$) are much faster than those determined by James and co-workers,[41] Pang and Stynes,[42] and Weschler et al.[43] using mixing techniques (Table V). Despite variations in heme, ligand, and solvent, a few general conclusions can be drawn. For imidazole, it appears that the activation energies of k^B_B and k^{CO}_B are roughly equal, because the ratio k^B_B/k^{CO}_B changes little with temperature. This is not the case for piperidine, where the k^B_B/k^{CO}_B ratio is 9 at −79 °C but 0.002 at 23 °C. This argues that the activation energy for k^B_B(pip) is greater than that for k^{CO}_B(pip). Using the data in Table V, it is possible to calculate a minimum E_{act} of 13 kcal mol^{-1} for k^{-CO}_B in both the imidazole and piperidine complexes. Similarly, loss of base from the bisimidazole heme, k^{-B}_B, has a higher activation energy (~16 kcal mol^{-1}) than does loss of base from bispiperidine heme (~7 kcal mol^{-1}). It therefore appears that not only rates, but also activation energies, are quite dependent on both ligand and macrocycle.

Table IV. Effect of Steric Constraint and Solvent on HBCO Rates[a]

ratio	k^{CO}_B	k^{-CO}_B	k^B_{CO}	k^{-B}_{CO}
(Im, benzene)/(2-MeIm, benzene)	12	0.062	1.1	0.017
(1-MeIm, CTAB)/(2-MeIm, CTAB)	12	0.066	1–5	0.01–0.07
(Im, benzene)/(1-MeIm, CTAB)	2.1	3.5	22	0.026
(2-MeIm, benzene)/(2-MeIm, CTAB)	2.1	3.8	20–100	0.02–0.1

[a] Deuteroheme dimethyl ester in benzene, mesoheme dimethyl ester in $CetMe_3NBr$.

The effects of solvent on HBCO rates and equilibria are shown in Tables IV and VI, respectively. The difference between the benzene and aqueous solutions arises not only from the intrinsic difference between the solvents but also from the stabilization of free imidazole in aqueous solution due to hydrogen bonding with the water and from the presence of water-liganded heme species.

The sensitivity of the CO rates and equilibria to solvent is due primarily to the presence of water-liganded species. The spectrum of reduced heme in CTAB micelles is very similar to that of reduced heme in benzene, indicating that the micellar heme is four coordinated. Addition of CO to the solution produces not HCO but $H(H_2O)CO$, as seen from the spectrum, which has a λ_{max} at 403 nm, similar to the 404 nm seen in aqueous benzene (HCO in dry benzene has a λ_{max} at 409 nm[16]). The observed K^{CO} in water is thus actually that for the $H \rightleftharpoons H(H_2O)CO$ equilibrium or $K^{CO}_{obsd} = K^{H_2O}K^{CO}_{H_2O}$. The binding of CO to the heme in the CTAB micelle has an equilibrium constant $K^{CO} = 4.7 \times 10^5$, which is greater than that in benzene alone, $K^{CO} = 4.5 \times 10^4$, but less than that measured for water-liganded heme in benzene, $K^{CO}_{H_2O} \simeq 4 \times 10^6$. In benzene the equilibrium constant for the addition of water to the heme, K^{H_2O}, is 0.1 M^{-1}.[16] Thus a calculated K^{CO}_{obsd}(benzene) = $K^{H_2O}K^{CO}_{H_2O}$ is 4×10^5 M^{-1}, very close to the measured value in the micelles.

In the measurement of CO rates, the presence of water affects not only the rate constants, but even which rates are measured by flash photolysis. Photolysis of $H(H_2O)CO$ at low [CO] gives a rate of return proportional to [CO]. If the effective concentration of water in the micelle is low, it is probable that the CO on rate measured is primarily that of four-coordinated heme $H \rightarrow HCO$ (the loss of water from $H(H_2O)$ presumably being fast compared to addition of CO). However, since the CO off rate is determined by division of the on rate by the equilibrium constant, the off rate k^{-CO} refers to $H(H_2O)CO$. The CO off rate depends strongly on solvent, k^{-CO}(benzene)/k^{-CO}(CTAB) = 33, presumably because

Table V. Heme-Base-CO Rates[a]

heme	ligand	solvent	temp, °C	k_B^{-B}	k_B^{-CO}	k_B^B/k_B^{CO}
PpIX	pip	toluene	23	20	0.06	0.002
TPP	pip	toluene	23	11	0.52	0.002
TPP	pip	CH_2Cl_2	−79	0.028	$<4 \times 10^{-6}$	9
TPP	1-MeIm	CH_2Cl_2	−79	6.1×10^{-4}	$<2 \times 10^{-7}$	20
DHD	Im	benzene	25	>1500	0.028	>8

[a] Values in toluene from ref 41a, in CH_2Cl_2 from ref 43, and in benzene from this work.

Table VI. Effect of Solvent and Steric Constraint on HBCO Equilibria[a]

ratio	K^B	K_B^{CO}	K_{CO}^B
(Im, benzene)/(2-MeIm, benzene)	0.35	195	66
(1-MeIm, CTAB)/(2-MeIm, CTAB)	0.40	180	73
(Im, benzene)/(1-MeIm, CTAB)	130	0.61	840
(2-MeIm, benzene)/(2-MeIm, CTAB)	150	0.55	930

[a] Deuteroheme dimethyl ester in benzene; mesoheme dimethyl ester in $CetMe_3NBr$.

k^{-CO} in water is in fact loss of CO from the hexacoordinated $H(H_2O)CO$. However, since CO does not hydrogen bond appreciably to water, and since the four-coordinated heme is the major species in the aqueous micelle, it is not surprising that the presence of water makes only a small difference in the CO on rate k^{CO}(benzene)/k^{CO}(CTAB) = 3. Similar small effects are found for the addition of CO to HB, k_B^{CO}(benzene)/k_B^{CO}(CTAB) = 2.1, and for the loss of CO from HBCO, k_B^{-CO}(benzene)/k_B^{-CO}(CTAB) = 3.5–3.8. (These last two are essentially independent of the steric hindrance of the base.)

The imidazole rates and equilibria show both the effects of water acting as a ligand for the heme and of water hydrogen bonding to the free imidazole. Water decreases the rate of addition of imidazole to the five-coordinated HCO at least in part because the latter exists as $H(H_2O)CO$ in the micelle, k_{CO}^{Im}(benzene)/$k_{CO}^{1\text{-MeIm}}$(CTAB) = 22. Water also increases the rate of loss of imidazole from HBCO, k_{CO}^{-Im}(benzene)/$k_{CO}^{-1\text{-MeIm}}$(CTAB) = 0.026, $k_{CO}^{-2\text{-MeIm}}$(benzene)/$k_{CO}^{-2\text{-MeIm}}$(CTAB) = 0.02–0.1. This result cannot be due primarily to hydrogen bonding of the bound imidazole N_3 nitrogen to water since the result is the same whether the nitrogen bears a hydrogen (2-MeIm) or a methyl substituent (1-MeIm). The effect must therefore be due to solvation of the free imidazole. This solvation is probably also the major reason for the increase in benzene solution of the addition of base to four-coordinated heme, K^B(benzene)/K^B(CTAB) ≃ 140.

In both benzene and aqueous solution, 2-MeIm binds better than does imidazole (or 1-MeIm) owing to the enhanced electron-donor properties of 2-MeIm. For imidazole in benzene, the equilibrium constant for addition of the second base, K_B^B, is more than an order of magnitude greater than that for addition of the first, K^B, while in aqueous suspension $K_B^B \simeq K^B$. In general low-spin six-coordinated iron complexes show a second affinity constant larger than the first. In aqueous suspension, any $H(H_2O)B$ will serve to lower the apparent value of K_B^B. Presumably this is only a minor portion of the large difference between K_B^B in benzene and CTAB, K_B^B(benzene)/K_B^B(CTAB) = 2100, because the equilibrium for addition of CO to HB is not affected by solvent.

The mechanisms discussed herein are relevant to a variety of heme-ligand kinetics measurements. In particular, studies dealing with the kinetics of four-coordinated hemes may be complicated by association of the solvent with the heme. Since the rate of ligand addition is a function of return to both four- and five-coordinated heme, this rate is dependent on the proportion of these two species. This proportion in turn is defined by the equilibrium constant for solvent association, K^S, and the concentration of the solvent, [S]. Therefore, differences in rates which appear to be a function of solvent, or of the electronic or steric properties of a four-coordinated heme, may in fact be due to differences in K^S or [S]. It must also be remembered that the CO rates for four-coordinated hemes are nearing diffusion control, and therefore that the rate of diffusion of the gas itself must be considered in viscous solvents, a problem treated by Hasinoff.[44] These problems are particularly acute in trying to compare model systems with heme proteins, for the mechanism of CO association in models depends on both the type and concentration of ligand (including solvent).

Table VII. Comparison of HBCO and Hemoprotein Rates and Equilibria

heme	k_B^{CO}, M^{-1} s^{-1}	k_B^{-CO}, s^{-1}	K_B^{CO}, M^{-1}
myoglobin[a]	5.0×10^5	0.017	2.9×10^7
T-state hemoglobin[b]	1×10^5	0.09	1×10^6
MHD, 2-MeIm, CTAB	4.8×10^5	0.12	4×10^6
DHD, 2-MeIm, benzene	1.0×10^6	0.45	2.2×10^6
R-state hemoglobin[c]	6×10^6	0.009	7×10^8
MHD, 1-MeIm, CTAB	5.8×10^6	0.008	7×10^8
DHD, Im, benzene	1.2×10^7	0.028	4.3×10^8
chelated mesoheme **1a**, CTAB[d]	1.1×10^7	0.015	7.3×10^8

[a] Horse myoglobin at 20 °C, ref 45. [b] k_B^{CO} from ref 5b, given here on a per heme basis, k_B^{-CO} from ref 5a. [c] k_B^{CO} from ref 5b; k_B^{-CO} from ref 5a. [d] References 15b and 33.

Heme Proteins. Of the various rates measured in this work, the rates of reactions of HB with CO are most obviously relevant to heme proteins, and are shown in comparison with hemoglobin[5] and myoglobin[45] in Table VII. Rate constants for the heme proteins are directly measurable, whereas those for heme-base-CO mixtures are obtained by extrapolation of measured rates to high base concentration. Nonetheless, the heme(1-MeIm) species in CTAB proves to be a good model for the reaction of R-state hemoglobin with CO. Heme(2-MeIm) serves as a model for T-state hemoglobin, adding CO more slowly and losing it more quickly than heme(1-MeIm). Both addition and loss of CO are somewhat faster in the model than in T-state hemoglobin, however. Utilization of benzene, rather than CTAB, as a solvent increases all of the rate constants, but does not alter the ratio between the Im and 2-MeIm species, indicating that the difference in rate constants is intrinsic to the heme-imidazole-CO system. From Table VII it can be seen that the ratio of the equilibrium constants for binding CO, $K_{Im}^{CO}/K_{2\text{-MeIm}}^{CO} \simeq 190$, is within a factor of 4 of that found in hemoglobin, Hb(R)/Hb(T) = 700. A similar situation has been found in oxygen equilibria measured for "picket fence" ferrous porphyrins, where the $P_{1/2}$ ratio $P_{1/2}$("chelated picket fence")/$P_{1/2}$(1,2-diMeIm) = 60[13] compared with Hb(R)/Hb(T) ≃ 40–600.[12b] (The range of ratios for hemoglobin reflects the sensitivity of the binding to phosphate and buffer concentration, the lower values being those in the absence of phosphate.)

The agreement between the models and the hemoproteins must be in part fortuitous since the two systems encompass changes in the porphyrin substituents and in the details of

geometry and solvation at the heme. However, it is apparent that the magnitude of steric constraint found in the external imidazole-heme system is a good model for the steric constraint found in hemoglobin, although the details of how this steric constraint arises must differ. In addition, the fact that models display both CO and O_2 binding similar to those of R- and T-state hemoglobin indicates that ligand binding is defined in large part by the ferrous porphyrin-imidazole system, and that it is therefore not necessary to invoke substantial steric constraint on the distal side of the heme in describing the bonding of CO. Although the effects described above have been attributed primarily to doming of the heme, and secondarily to the electronic nature of the Fe-imidazole bond, it is possible that the rotation and tilt of the imidazole plane with respect to the heme may also influence the rates of ligand binding. These factors have not been considered in detail, both because values measured by X-ray may be a function of intermolecular packing in the crystal and because there is not enough data to do justice to such a discussion.

Myoglobin is not modeled well by either heme(Im) or by heme(2-MeIm). Mb adds CO with a rate constant similar to heme(2-MeIm) but loses CO with a rate constant similar to heme(Im). This discrepancy may be due to a distal side effect; the problem is under investigation.

This work, and previous studies of the model hemes bearing covalently attached bases,[15] indicates that strain between the proximal base and heme plane is reflected kinetically not only in the slower rates associated with the hindered base, but also in a switchover of the primary mode of CO association from addition to the five-coordinated HB to addition to the four-coordinated H, or the base-elimination pathway. The fraction f of the reaction proceeding through the four-coordinated pathway is dependent upon both the concentration of imidazole and the concentration of carbon monoxide. At low base and low CO concentration loss of base from HB is fast with respect to addition of CO to HB, and addition of CO to the four-coordinated heme, H, is fast with respect to addition of B to H ($k^{-B} > k_B^{CO}[CO]$ and $k^{CO}[CO] > k^B[B]$), and the overall reaction proceeds through base elimination. At low [CO] and high [B] the base equilibria are established before CO addition and the fraction proceeding by base elimination is given by

$$f = \frac{k^{CO}}{k^{CO} + k_B^{CO} K^B [B]} \qquad (13)$$

At high [CO] and high [B] the reaction depends upon the reaction of HB after the flash, e.g., on the relative rates of $k_B^{CO}[CO]$ and k^{-B}; the fraction will be, at maximum, the f given above, and will be less than f if $k^{-B} \leq k_B^{CO}[CO]$. These points are illustrated in Figure 7, which shows a plot of the percentage of base elimination and direct association as a function of the concentration of base for [CO] = 1×10^{-5} M. For any given concentration of base, Im proceeds via the direct addition pathway more than does 2-MeIm, reflecting the fact that CO adds to heme(Im) faster than it does to heme(2-MeIm). Also, for any concentration of base, more direct association is seen in benzene than in CTAB, reflecting the higher concentrations of HB (larger K^B) in the former solvent.

Utilization of the base-elimination pathway in heme proteins would depend on the K^B equilibrium constant, the effective molarity of the proximal histidine, the rate of loss of the proximal histidine, and the difference in rates between ligand addition to the four- and five-coordinated heme. While it is unlikely that the base-elimination mechanism plays a role in the physiological function of hemoglobin or myoglobin, there are experiments which provide evidence for the lability of the heme-histidine bond.

Flash photolysis of CO-ligated myoglobin as a function of pH revealed a rate increase with decreasing pH.[46] In a stopped-flow apparatus, it was possible to obtain k_{obsd}^{CO} before

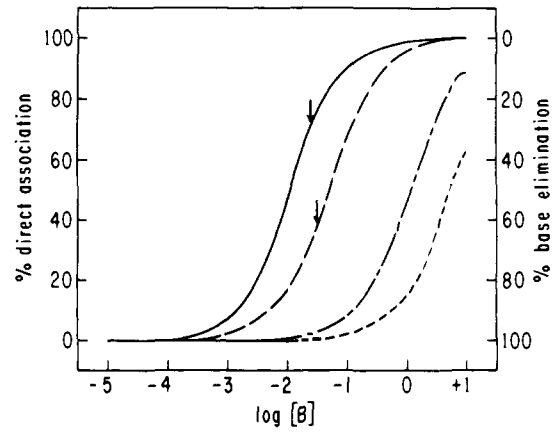


Figure 7. The percentage of direct association of CO as a function of the concentration of external base, [CO] = 1×10^{-5} M. Arrows indicate the maximum solubility of the base: (—) Im in benzene; (---) 2-MeIm in benzene; (-·-) 1-MeIm in CTAB; (·····) 2-MeIm in CTAB.

denaturation, and a pH-rate profile showed a 32-fold increase in rate from pH 8 to 2, as would be expected if the proximal imidazole were being protonated, and the CO addition therefore proceeding via a base-elimination pathway. Similarly a pH-rate profile on "chelated heme" **1a** showed a 30-fold rate increase over the same pH range.[14a,46] The rates reported herein indicate a 41-fold difference between four-coordinated and unstrained five-coordinated rates of addition of CO.

A second line of evidence comes from the reaction of horseradish peroxidase compound II (HRP-II) with iodide ion:[47]

$$\text{HRP-II} + I^- \rightarrow \text{HRP} + \tfrac{1}{2}I_2$$

The logarithm of the second-order rate constant increased linearly with decreasing pH from −1.0 (0.1 M^{-1} s^{-1}) at pH 9.0 to 5.4 (2.3×10^5 M^{-1} s^{-1}) at pH 2.7. This pH dependence was explained in terms of an acid dissociation outside the pH range of the study, perhaps due to protonation of the proximal histidine of HRP-II.[47b]

Further evidence on the lability of the histidine-Fe bond comes from studies of nitrosylhemoglobins. In the absence of IHP, nitrosylhemoglobin A, like other low-spin compounds of hemoglobin A, exists in the oxy or R state. Addition of IHP causes a transformation to the deoxy, or T, structure, as first seen by Cassoly[48] and by Salhany.[49] This R → T switch is followed by a slower process which occurs within the quaternary deoxy structure. The molecular basis of this slower change was elucidated by Maxwell and Caughey's study of the NO stretching frequencies in nitrosylhemoglobin.[50] They found that nitrosylhemoglobin A without IHP exhibited a single ^{14}NO stretching frequency (1615 cm^{-1}) and that addition of IHP caused the intensity of this band to decrease by half and a new band (1668 cm^{-1}) to appear. Comparison with model five- and six-coordinated hemes led Perutz et al.[51] and Maxwell and Caughey[50] to suggest that these two bands corresponded to two species, the first the six-coordinated N_ϵ-Fe-NO and the second a species in which the bond to the proximal histidine was either broken or severely stretched.

This hypothesis has been supported by ESR[52] and Raman data,[53] and by studies of hemoglobin variants and hybrids.[51,54] The rate of change has been studied by measurement of the optical[55] or ESR[56] spectra of solutions produced by mixing either $Hb_4(NO)_4$ with IHP or Hb_4IHP with NO giving rates of 1-6 s^{-1}. Our results for the T-state model give values of 150 s^{-1} in benzene and $2\text{-}7 \times 10^3$ s^{-1} in CTAB for loss of 2-MeIm from HBCO. These might indicate that the observed rate in nitrosylhemoglobin is not loss of base, but rather a conformational change following loss of base.

Conclusions

The idea that strain at the heme site is the molecular basis of cooperativity has been investigated both by using spectroscopic techniques to search for strain at the heme in hemoglobin itself and by measuring the kinetics and equilibria of ligand binding to strained and unstrained heme models. Spectroscopic studies generally give little or no evidence for strain, and yet models mimic R- and T-state hemoglobin well. It appears that a small change in heme geometry is enough to effect a large change in reactivity.[57] In other words, the important factor in the T to R switch in hemoglobin may not be the strain of the five-coordinated heme, but the difference in this strain between five- and six-coordinated heme.[11,13]

We have shown that both strained and unstrained heme-imidazole complexes react with carbon monoxide predominantly by the base-elimination mechanism. Extrapolation of the kinetic behavior to infinitely high base concentration allows the rate constants of the direct-association mechanism to be estimated. Under these circumstances the effect of strain is seen to reduce the carbon monoxide on rate and increase its off rate just as in the case of the R- to T-state change in hemoglobin. These results and our previous finding that oxygen off rates are increased by strain (reducing oxygen affinity) provide support for the Perutz strain mechanism. Our results also suggest that the base-elimination pathway is important in heme reactivity, and that this mechanism should be sought in heme proteins.

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(27) All rates and equilibria in this work are expressed in terms of the concentration of CO, rather than its partial pressure over the solution, in line with the work of Rougee and Brault.[16] This allows us to compare samples prepared in tonometers with a free space over the solution (addition of gaseous CO) with those prepared in "closed" cuvettes entirely filled with solution (addition of CO-saturated solvent). In addition, it allows accurate determination of the concentration of CO in solution after the flash, which is the sum of the free CO in solution and the CO flashed off the heme.
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