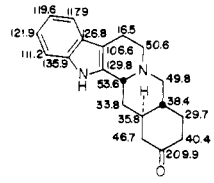

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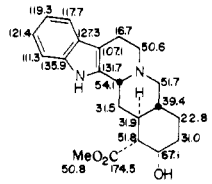

22

h. More catalyst, 10 mg, was added and the hydrogenation continued for another 24 h. The catalyst was filtered and washed with methanol and the combined washings and filtrate evaporated. The residue was taken up in 20 mL of methylene chloride and the solution treated with 5 mL of 5% sodium bicarbonate solution, washed, dried, and evaporated. A methylene chloride solution of the residue, 55 mg, was chromatographed on 3 g of silica gel. Elution with 100:1 ether–methanol gave 47 mg (72%) of solid, a single spot on TLC with various solvent systems, whose crystallization from methanol yielded (±)-pseudoyohimbine (**13**), mp 249–251 °C dec (lit. mp[1a] 252–256 °C, charring at 250 °C; mp[1c] 248–251 °C).

Acknowledgment. The authors express their gratitude to the U.S. Public Health Service for support of this work.

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Kinetics and Mechanisms of Carbon Monoxide Dissociation from Chelated Heme–CO Complexes. Models for Hemoprotein Reactions[1a]

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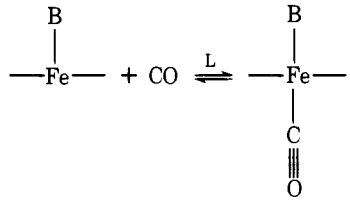
Abstract: Rates of carbon monoxide dissociation from a series of chelated heme compounds have been measured. Unstrained chelated hemes, having attached proximal bases, remain five coordinated and therefore react reversibly with carbon monoxide by a simple one-step mechanism. Both the rates and activation enthalpies of dissociation are similar to those of fully ligated (R-state) hemoglobin, indicating that chelated protoheme accurately models hemoglobin in its R state. This leads to the conclusion that the iron–carbon–oxygen bending observed in carboxyhemoglobin does not appreciably affect the CO off rates or affinities. Variations in side-chain electron donation or in proximal basicity cause no appreciable change in the CO dissociation rate. Solvent polarity is also without substantial effect. However, introducing steric strain in the proximal base chelating arm causes the mechanism of dissociation to change from direct dissociation to base elimination.

Although carbon monoxide is not a natural substrate in biological systems, its interaction with metalloproteins has been widely studied.[2-6] This interest in carbon monoxide derives from its strong binding to those metalloproteins which bind or utilize oxygen, and its consequent ability to interfere with or inhibit their function. In those hemoproteins which contain a five-coordinated ferrous ion, CO binding constants vary greatly: $L \simeq 10^5\ M^{-1}$ for cytochrome P-450[7] to $L \simeq 7 \times 10^8\ M^{-1}$ for hemoglobin.[2b,8] This wide variation in binding constants as well as some variation in the spectroscopic properties of the carbon monoxide complexes has made carbon monoxide binding an important tool in the characterization of hemoproteins.



(1)

To understand the structure-reactivity relationships developed in natural and reconstituted hemoproteins[3-5] similar structure-reactivity relationships in simple five-coordinated hemes which resemble the hemoprotein active sites are required. To fill this need we have prepared a number of chelated

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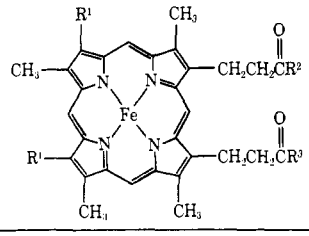
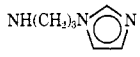
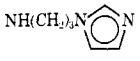
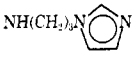
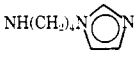
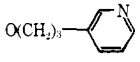
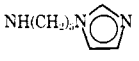
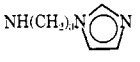
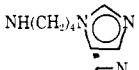
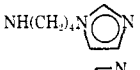
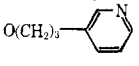
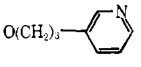
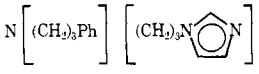
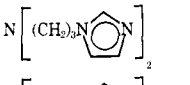
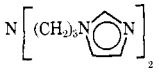
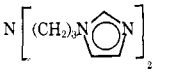
Table I. Structures of Heme Compounds[a,b]



compd	R^1	R^2	R^3	ref
1, mesoheme dimethyl ester	Et	OCH_3	OCH_3	9g
2, chelated mesoheme	Et	OCH_3		9g
3, chelated diacetyl-deuteroheme	$CH_3C{=}O$	OCH_3		9i
4, chelated protoheme	$CH_2{=}CH$	OCH_3		9g
5, C_4-chelated mesoheme	Et	OCH_3		9e
6, pyridine chelated mesoheme	Et	OCH_3		9g
7, dichelated mesoheme	Et			9e
8, di-C_4-chelated mesoheme	Et			this work
9, dipyridine chelated mesoheme	Et			9e
10, phenyl forked heme	Et	OCH_3		this work
11, forked heme	Et	OCH_3		9e
12, diforked heme	Et			9e

[a] These compounds are a 1:1 mixture of the isomer shown and that where R^2 and R^3 are inverted; however, the mixtures are kinetically homogeneous. See ref 9e and 9g for the syntheses. [b] As specified previously,[9e] the number, e.g., **9**, refers to the Fe^{II} five-coordinated species, **9**$^+Cl^-$, the Fe^{III} species, **9**-P, the free porphyrin, etc.

heme compounds having the proximal base covalently attached to the heme through a side chain.[9a,b] This side chain can be designed to allow strain-free association of the proximal base (the chelation arm) with the iron.[9c] Five coordination is therefore assured by the neighboring-group effect.[9d] Alternatively, strained chelation arms can be attached.[9e]

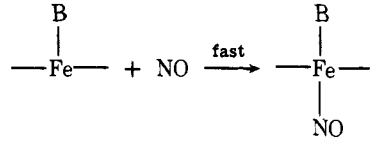
In this paper we attempt to answer questions posed in the hemoprotein work: What are the effects of electron donation, proximal basicity, proximal base strain, and solvent polarity on the rates of single-step dissociation of carbon monoxide to produce five-coordinated hemes? We describe the kinetics of dissociation of carbon monoxide from a series of chelated heme-carbon monoxide complexes, establish the effect of structure and solvent change on this dissociation, and present definitive evidence for the change in CO dissociation mechanism from direct dissociation to the base-elimination mechanism[9f] as strain is introduced in the complex.

Results

The chelated heme compounds having covalently attached proximal base arms studied in this work are shown in Table I. The syntheses and characterization of these compounds are described elsewhere.[9e,g]

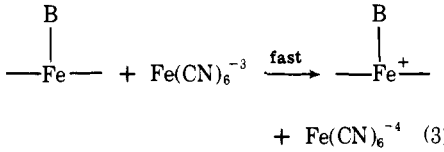
Validity of the Kinetic Methods. The loss of carbon monoxide from a heme-CO complex is accompanied by a very large change in the position and absorbance of the Soret band around 400 nm, and this affords a convenient and accurate method of observing this rate.[2a,6a] However, because the dissociation is usually slow ($l = 10^{-2}$–$5\ s^{-1}$ in this study) and the association fast ($l = 10^5$–$10^8\ M^{-1}\ s^{-1}$), the recombination must be prevented. This has been accomplished in hemoprotein studies by mixing the carbonmonoxy hemoprotein with the

heme trap method:



(2)

heme oxidation method:



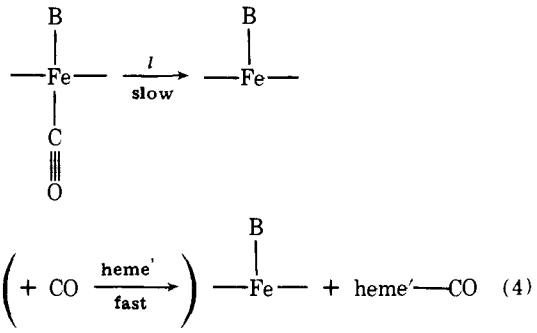
(3)

Table II. Kinetics of Carbon Monoxide Dissociation from Carbonmonoxy Chelated Protoheme in 80/20 (v/v) Methanol-Water. A Comparison of Methods

temp, °C	slit width, mm	concn of 4-CO, μM	concn of CO trap, 3[a], μM	ratio	l, s^{-1} [b]
22 ± 0.5	0.5	1.5	53	35	0.046
22 ± 0.5	0.5	1.5	37.8	25	0.042
22 ± 0.5	0.5	1.5	25.7	17	0.040
22 ± 0.5	0.5	1.5	15.2	10	0.042
22 ± 0.5	0.5	1.5	10.7	7.1	0.044
22 ± 0.5	0.5	1.5	6.8	4.5	0.040
22 ± 0.5	0.5	1.5	3.95	2.6	0.035
31 ± 0.5	0.5	3.6	26.7	7.3	0.13
31 ± 0.5	0.25	3.6	26.7	7.3	0.13
31 ± 0.5	1.0	3.6	26.7	7.3	0.14
31 ± 0.5	3.0	3.6	26.7	7.3	0.14
20 ± 0.2	0.25	2.4	11.5		0.029
20 ± 0.2	0.25	5			0.029[c]

[a] Concentrations before mixing equal volumes in the stopped-flow apparatus. [b] Each rate is an average of three to eight runs, ±10%. [c] Determined by mixing the heme-CO solution (~5 μM) with 50-200 μM $K_3Fe(CN)_6$ solutions. All of the compounds **2**–**12** showed no dependence of the observed rate on the concentration of ferricyanide.

CO trap method:



rapidly reacting NO (eq 2)[10] or by oxidizing the heme with ferricyanide ion (eq 3),[11] both of which trap the free heme. We recently introduced a method which employs a second heme to bind the CO as it dissociates, the CO-trap method (eq 4).[6a]

In aqueous methanol solutions of chelated hemes, ferricyanide reacts with the deoxyheme within the dead time of the Durrum stopped-flow instrument even at 5 μM ferricyanide concentration, indicating a second-order rate that is near diffusion control. In addition, the observed off rates are independent of ferricyanide concentration from 20 to 200 μM (see Table II). The rate-limiting step in this oxidation (eq 3) is therefore CO dissociation.

The requirements of the CO-trap method are that there be large spectral changes accompanying reaction 4 and that this reaction be essentially irreversible. In this work we have chosen three compounds as the trapping hemes: chelated mesoheme (**2**), λ_{max}^{CO} 410 nm, chelated protoheme (**4**), λ_{max}^{CO} 420 nm, and chelated diacetyldeuteroheme (**3**), λ_{max}^{CO} 439 nm. Corresponding differences in the spectra of the deoxy and oxidized forms of these compounds are also observed. Thus, when an aqueous cetyltrimethylammonium bromide ($CetMe_3NBr$) solution of carbonmonoxy chelated protoheme (**4**-CO), λ_{max} 420 nm, is mixed in a stopped-flow apparatus with an excess of chelated diacetyldeuteroheme (**3**), λ_{max} 449 nm, the 420 and 449 nm peaks of **3** and **4**-CO are replaced by 439- and 430-nm peaks of **3**-CO and **4**, respectively.

As it turned out the carbon monoxide affinities and on rates for **2**, **3**, and **4**, are similar,[9i] so that when an excess of the CO-trap heme was used, first-order rates were observed. The effect of the ratio of the trap, **3**, to the CO complex, **4**-CO, on the observed off rate is shown in Table II. All ratios above five produced the same observed rate. This table also shows the independence of rate on (narrow) slit width, indicating no interference of the rather facile photodissociation of **4**-CO. The last two entries also indicate that the oxidation method and the CO-trap method give the same results.

Table III. Rates of Carbon Monoxide Dissociation from Heme-CO Complexes at 20 ± 0.2 °C in 80/20 (v/v) Methanol-Water

compd	method[a]	l, s^{-1} [b]
2-CO	$Fe(CN)_6^{3-}$	0.059
3-CO	$Fe(CN)_6^{3-}$	0.016
4-CO	$Fe(CN)_6^{3-}$	0.029
4-CO	trap	0.029
5-CO	$Fe(CN)_6^{3-}$	0.033
6-CO	$Fe(CN)_6^{3-}$	0.034
7-CO	$Fe(CN)_6^{3-}$	0.051
8-CO	$Fe(CN)_6^{3-}$	0.042
9-CO	$Fe(CN)_6^{3-}$	0.032
10-CO	$Fe(CN)_6^{3-}$	0.30
10-CO	trap	0.26
11-CO	$Fe(CN)_6^{3-}$	0.13
11-CO	trap	0.13
12-CO	$Fe(CN)_6^{3-}$	0.068
12-CO	trap	0.058

[a] See text. [b] Average of two to eight runs, ±10%.

Effects of Structure on Dissociation Rates. The dissociation rates of the series of chelated heme-CO complexes **2**-CO–**12**-CO, determined in an 80/20 (v/v) methanol-water mixture, are shown in Table III. A similar series, determined in aqueous suspension in either 2% $CetMe_3NBr$ or in a mixture of $CetMe_3NBr$ and myristyltrimethylammonium bromide ($MyrMe_3NBr$), is shown in Table IV.

The first striking effect in Table III is the insensitivity of CO dissociation to electronic effects. While in both methanol-water and in aqueous suspension the two ethyl groups (**2**-CO) seem to increase l somewhat, there is no appreciable effect of two acetyl (**3**-CO) groups as compared with two vinyl (**4**-CO) groups. Similarly the proximal pyridine compound (**6**-CO) dissociates at about the same rate (0.035 s^{-1}) as do the imidazole compounds **2**-CO (0.019 s^{-1}) and **5**-CO (0.024 s^{-1}). The conclusion is that neither side chain nor proximal base electronic effects alter the dissociation rates very much.

Solvent Effects on Dissociation. Table V lists rates of CO dissociation in a series of solvents which range from highly polar to highly nonpolar. The clearest indication of polarity effects is seen in the H_2O-MeOH-toluene series. Interestingly, from 50% aqueous methanol to 95% toluene there seems to be no change in carbon monoxide dissociation rate. Such an in-

Table IV. Rates of Carbon Monoxide Dissociation from Heme-CO Complexes in 2% $CetMe_3NBr$, 0.15 M Phosphate Buffer at pH 7.3, 20 ± 0.2 °C

compd	method[a]	l, s^{-1} [b]
2-CO	$Fe(CN)_6^{3-}$	0.019
3-CO	$Fe(CN)_6^{3-}$	0.0063
4-CO	$Fe(CN)_6^{3-}$	0.0052
4-CO	trap	0.0079
4-CO	trap	0.015[c,d]
4-CO	trap	0.016[d]
5-CO	$Fe(CN)_6^{3-}$	0.024[e]
6-CO	$Fe(CN)_6^{3-}$	0.035
7-CO	$Fe(CN)_6^{3-}$	0.015
10-CO	trap	0.073[c]
11-CO	trap	0.053[c]
12-CO	trap	0.027[c]

[a] See text. [b] Average of two to seven runs, ±10%. [c] The suspending agent was 1.6% $CetMe_3NBr$ and 0.4% $MyrMe_3NBr$. [d] Temperature 25 ±0.1 °C. [e] The $Fe(CN)_6^{3-}$ runs used 0.05 M buffer.

sensitivity to solvent polarity suggests that there is little charge separation in the heme-CO bond.

Temperature Effects. Kinetics of dissociation of chelated protoheme-CO (**4**-CO) were determined in both methanol-water mixtures and in aqueous suspension. The data are given in Table VI. Plots of k/T vs. $1/T$ reveal $\Delta H^{\ddagger} = 25.0 \pm 0.6$ kcal/mol, $\Delta S^{\ddagger} = 17 \pm 2$ eu in aqueous suspension and $\Delta H^{\ddagger} = 23.7 \pm 0.4$ kcal/mol, $\Delta S^{\ddagger} = 16 \pm 1.2$ eu in methanol-water.

Discussion

The CO-Trap Method. The principle of the CO-trap method is that as a carbon monoxide dissociates it is rapidly and irreversibly trapped by another heme to produce a new CO complex with a different spectrum. It has the advantage of being adaptable to any solvent system and of being a true dissociation rather than a displacement process. It has the disadvantage of being reversible and therefore being an approach to equilibrium. However, it is possible to choose conditions under which the reaction is irreversible within our experimental error. Consider the dissociation of HmCO using the second heme H as the trap.

$$\mathrm{HmCO} \underset{l'}{\overset{l}{\rightleftharpoons}} \mathrm{Hm} + \mathrm{CO} \tag{5}$$

$$\mathrm{H} + \mathrm{CO} \underset{l^*}{\overset{l''}{\rightleftharpoons}} \mathrm{HCO} \tag{6}$$

$$-\frac{d\mathrm{HmCO}}{dt} = l_{obsd}(\mathrm{HmCO}) = \frac{l(\mathrm{HmCO})l''(\mathrm{H}) - l^*(\mathrm{HCO})l'(\mathrm{Hm})}{l''(\mathrm{H}) + l'(\mathrm{Hm})} \tag{7}$$

The observed rate constant, l_{obsd}, will be equal to l when $l''(\mathrm{H}) \gg l'(\mathrm{Hm})$ and $l \geq l^*$. This situation can be achieved with a trapping heme, H, having a large on rate constant, l'', or if $l'' = l'$, as in the present study, by using large concentration ratios $\mathrm{H} \gg \mathrm{Hm}$. Table II indicates that using chelated diacetyldeuteroheme (**3**) as the CO trap (H) the observed reaction rate is not changed by altering the ratio of **3** to **4**-CO from 4.7 to 35. The small inaccuracy (~10%) expected for the lowest ratio is within our run-to-run experimental error. Even in those cases where the carbon monoxide binding constants of the heme under study and the CO-trap heme are equal, this method still gives accurate dissociation rates when excess trap is used. In this way **3** can be used as a trap for **2**-CO and **2** can be used as a trap for **3**-CO. These results, and the fact that we can prepare CO-trap chelated hemes with any desired solubility by attaching, e.g., a hydrocarbon or polyglycol tail,[12] make the CO-trap method the most versatile method available for determination of carbon monoxide dissociation rates.

Table V. Rates of Carbon Monoxide Dissociation from Chelated Protoheme-CO (**4**-CO) Complexes in Various Solvents at 20.0 ± 0.1 °C

solvent	method[a]	l, s^{-1} [b]
MeOH	trap	0.022
MeOH	$Fe(CN)_6^{3-}$(crown)[c]	0.022
80/20 (v/v) MeOH-H_2O	$Fe(CN)_6^{3-}$	0.029
80/20 (v/v) MeOH-H_2O	trap	0.029
50/50 (v/v) MeOH-H_2O	$Fe(CN)_6^{3-}$	0.032
50/50 (v/v) MeOH-toluene	$Fe(CN)_6^{3-}$(crown)[c]	0.029
5/95 (v/v) MeOH-toluene	trap	0.025

[a] See text. In each case where $Fe(CN)_6^{3-}$ was used, variation of its concentration from 50 to 200 μM caused no change in rate. [b] Average of two to eight runs, ±10%. [c] The $K_3Fe(CN)_6$ was rendered soluble with an equivalent concentration of 18-crown-6.

Table VI. Effect of Temperature on CO Dissociation from Chelated Protoheme-CO (**4**-CO)

temp,[a] °C	l, s^{-1} [b] $CetMe_3NBr$-$MyrMe_3NBr$[c]	l, s^{-1} [b] MeOH-H_2O[d]
10		0.0061
15		0.014
20	0.0089	0.030
25	0.014	0.050
30	0.032	0.11
35	0.067	0.22
40	0.13	0.47
45	0.26	0.80
50	0.47	1.21
$\Delta H^{\ddagger}$, kcal/mol	25.0 ± 0.6	23.7 ± 0.4
$\Delta S^{\ddagger}$, eu	17 ± 2.5	16 ± 1.2

[a] Temperature maintained ±0.2 °C. [b] Rate calculated from the first two half-lives, ±10%. [c] $CetMe_3NBr$ (1.6%)-$MyrMe_3NBr$ (0.4%) mixture in 0.15 M phosphate buffer at pH 7.3; 2.1 μM **4**-CO and 11.5 μM **3** before mixing. [d] 80/20 v/v MeOH-H_2O, 2.7 μM **4**-CO and 11.5 μM **3** before mixing.

Comparison of Chelated Hemes with Hemoproteins. The CO dissociation rates of R-state hemoglobin and hemoglobin isolated α chains are 0.009[6b] and 0.015 s^{-1},[2c] respectively, at 20 °C. The dissociation of carbonmonoxy chelated protoheme (**4**-CO) has a comparable rate constant of 0.008 s^{-1} in aqueous $CetMe_3NBr$ suspension. Furthermore, the activation enthalpy for this dissociation in aqueous suspension is 25 kcal/mol compared to 23 to 25[2d,6b,8b] kcal/mol for R-state hemoglobin. These results further document our previous conclusions that chelated hemes in aqueous suspension closely duplicate the behavior of R-state hemoglobin.[9e,g] Although change of solvent from aqueous suspension to methanol-water alters the rate constant slightly ($l = 0.029$ s^{-1}, $\Delta H^{\ddagger} = 23.8$ kcal/mol), the comparison with R-state hemoglobin, etc., is still quite good.

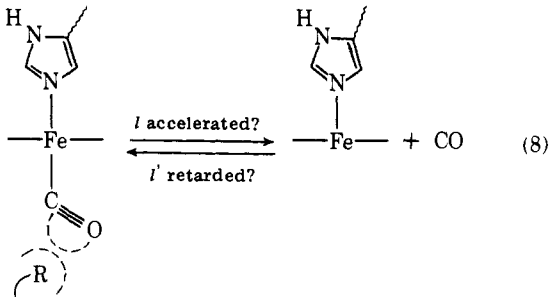
Our results contrast the reports that crystalline "picket fence" heme mimics the dioxygen binding to R-state hemoglobin but not the corresponding CO binding.[13a] Chelated protoheme (**4**) mimics both of these reactions equally well.[9h]

Because several different heme model compounds[9g,h,14] bind CO with affinities similar to those of chelated protoheme and it in turn resembles R-state hemoglobin, we see no reason for the apparent "irreversible" binding of CO to the crystalline picket fence heme mentioned above. Perhaps diffusion is severely limited in a solid by the usual high affinity of each site for CO. We have been unable to remove CO from a solid film

of a chelated heme by evacuation at room temperature,[9a] although the CO-binding constant is quite ordinary.[9a,b]

There also appears to be an impression[13b,15] that model compounds in general bind CO more strongly than do hemoproteins due to some distal side steric interference which operates for CO but not for dioxygen.[16] But the closest electronic model for R-state hemoglobin, chelated protoheme **4**, binds CO with the same association[9g] and dissociation constants as does R-state hemoglobin. Thus our data exclude such steric effects in R-state hemoglobin and we have shown elsewhere[9h] that proximal 2-methylimidazole-heme complexes model the CO binding[9h] of T-state hemoglobin exactly as well as they model the dioxygen binding.[13b] We therefore find no evidence from model compound studies for the suggestion[16a,13b] that CO and O_2 are differentiated with regard to steric effects in hemoglobin.

These results also bring into question the proposals that the bent Fe-CO bond in hemoproteins accounts for variations in CO affinity.[13,15] Such steric crowding with organic compounds



(8)

has been studied in great detail. Invariably, steric crowding of the leaving group results in increased dissociation rates.[17] Because R-state carboxyhemoglobin shows no such increased dissociation rate relative to carboxy chelated protoheme even though the Fe-CO bond in this protein is bent to the same extent as in other hemoproteins, we conclude that the bending reported in hemoproteins has little effect on CO dissociation rates. However, it is possible that the distal imidazole or another group affords the same extent of steric interaction to bend the Fe-CO bond in the starting Fe-CO complex and in the transition state for dissociation. In this circumstance the CO dissociation rate would be unaffected by the steric hindrance but the association rate would be retarded, resulting in reduced affinity. Our results also agree with the conclusions of Caughey[16c] that there is no general relationship between stretching frequencies in heme-CO complexes and carbon monoxide affinities. While CO stretching frequencies depend upon proximal base and side-chain electronic effects as well as solvent type,[16c] we find dissociation rates to be insensitive to these changes. The CO stretching frequency for **4**-CO is 1951 cm^{-1} in dimethyl sulfoxide,[9j] identical with that of hemoglobin R state, and about 1965 cm^{-1} in chloroform. This, and the small polarity effect on CO affinity, suggests that the CO stretching frequencies in hemoproteins could be altered by orientation changes in the polar distal imidazole without changing the CO affinity.

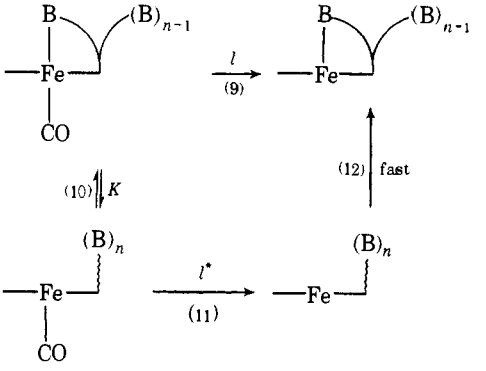
We propose that variations in carbon monoxide off rates in hemoproteins having a proximal imidazole are due to proximal side strain and that variation in carbon monoxide association rates can be due to either proximal side strain or some steric interference with CO at positions in the heme pocket which are perhaps remote from the iron. This would correspond to one or more of the outer barriers to CO binding described for myoglobin by Frauenfelder and his co-workers.[18] The observation that myoglobin has a slower CO association rate than does hemoglobin α chain but a comparable dissociation rate[2d,e] is consistent with this theory.

Solvent Effects. The endothermic dissociation of carbon monoxide produces two species which react rapidly ($k \simeq 10^7$ M^{-1} s^{-1})[9b] and therefore has a transition state which resembles the dissociated product. The effect of solvent polarity on this dissociation rate appears to be negligible. Consider the effect of changing the solvent from 95% toluene-5% methanol to 50% methanol-50% water on the ionization of, e.g., *tert*-butyl chloride. An acceleration of $>10^5$ in rate would be expected.[17] The CO dissociation rates in these two solvents are identical. We conclude from this result that the polarity of the Fe-CO bond is about the same as the polarity of the separated species. This is consistent with the electronic effects discussed above and contrasts to the large solvent effect which we observed with dioxygen binding.[9c] Our results are inconsistent with the suggestion that the solvent effects on dioxygen binding are due to some interaction of solvent with the dissociated heme.[13b] If this were the case then carbon monoxide binding should display solvent effects like those of dioxygen. However, both O_2 and CO dissociate about three times more slowly in aqueous suspension than in polar organic solvents, and this could relate to changes in the activity of these gases.

Mechanisms of Carbon Monoxide Dissociation. We have previously presented kinetic,[9c] pH-rate profile,[9f] and flash spectroscopic evidence[9e,g] for the intermediacy of a five-coordinated heme-CO species in the reaction of base-heme or base$_2$-heme with CO. This requires the same HmCO intermediate during CO dissociation.

We now present dissociation rate data which corroborates our previous conclusions that our R-state (unhindered) chelated heme models react by direct dissociation of CO whereas the forked chelated heme models,[9e] which apparently react by direct dissociation of dioxygen at rates approaching those of T-state hemoglobin, nevertheless react predominantly by base elimination during CO dissociation.

Consider the following mechanisms of CO dissociation from chelated hemes having one, two or four chelation arms. The dissociation rate expressions for direct dissociation (9) and base



elimination (10-12) are

$$\frac{dCO}{dt} = l[\text{heme-CO}] \quad (13)$$

$$\frac{dCO}{dt} = K_{10}^{-1} l^* [\text{heme-CO}] \quad (14)$$

Clearly the direct dissociation mechanism requires the observed rate to be independent of the equilibrium constant K_{10}, whereas base elimination requires that the rate be inversely proportional to K_{10}. The results are tabulated in Table VII for structures shown in Figure 1.

The observed rates for the unstrained pairs **2**-CO/**7**-CO and **6**-CO/**9**-CO are the same within experimental error, even though the first member of each pair has one chelation arm and the second, two. The mechanism of CO loss is therefore direct dissociation.

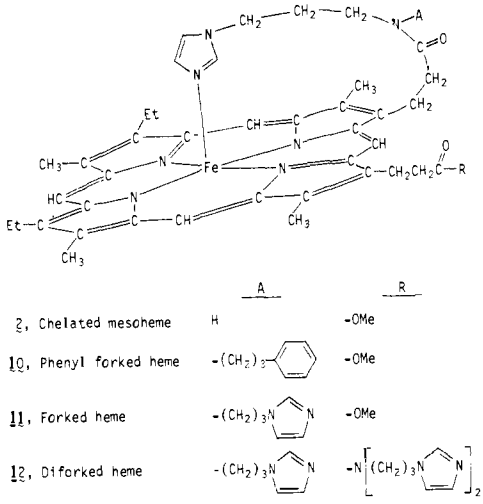


Figure 1. Forked hemes.

Table VII. Rate Constants for Dissociation of Carbon Monoxide from Carbonmonoxy Chelated Hemes at 20 °C in 80% (v/v) Methanol-Water

compd	no. of imidazole arms (n)	l, s^{-1}
2-CO, chelated mesoheme	1	0.059
7-CO, dichelated mesoheme	2	0.051
6-CO, pyridine chelated mesoheme	1 (pyridine)	0.034
9-CO, dipyridine chelated mesoheme	2 (pyridines)	0.032
10-CO, phenyl forked mesoheme	1	0.28[a]
11-CO, forked mesoheme	2	0.13[a]
12-CO, diforked mesoheme	4	0.063[a]

[a] Average of runs by both methods from Table III.

A different behavior is found for the compounds **10**-CO, **11**-CO, and **12**-CO, which bear one, two, and four imidazoles, respectively. Here each doubling of the number of imidazoles decreases the observed off rate by a factor of 2. The dependence of the off rate on the number of imidazoles (e.g., on K) shows that the main dissociation pathway is one of base elimination. Thus, although **7** and **11** appear similar in that each carries two imidazoles which can bind to the heme, they behave very differently in their reactions with CO.

The strain in **11** is due to the difficulty of accommodating a large alkyl substituent on the amide nitrogen of the chelation ring, discussed in more detail below. Although the kinetic data on carbonmonoxy chelated protoheme, **4**-CO, indicate it to be unstrained (compare **2** with **5**), the NMR spectrum[9g] shows the chelation ring to be highly structured. The substitution of an alkyl group for the amide NH (forked at N) causes this chelation arm to be forced away from the heme (see Figure 1).

This strain is documented in the spectra of the deoxy forms of the forked hemes. The spectra of four-, five- and six-coordinated mesoheme derivatives are definitive for ligation.[9e,14b] Compare in Figure 2 the spectra of the four-coordinated mesoheme **1** with the five-coordinated **2** and six-coordinated **8**. A peak at 525 nm in **10** is indicative of some four coordination and a corresponding shoulder in **11** indicates less four-coordinated heme than in **10**. Comparisons of the spectra of **12** with that of **8** indicate that **12** is almost completely six coordinated. These spectra agree with the kinetic data showing that chelated forked hemes are strained and that the effect of this strain on the chelation equilibrium constant K (reaction 10) can be overcome by increasing the concentration of the attached imidazoles. The spectra of the carbon monoxide derivatives **10**-CO, **11**-CO, and **12**-CO are identical with those of **2**-CO

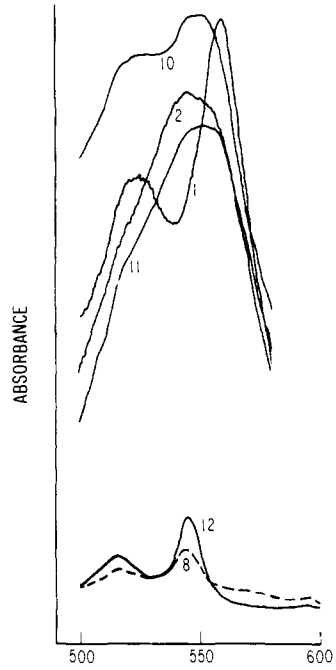


Figure 2. Visible spectra of deoxyheme compounds at ~5 μM concentration in 2% $CetMe_3NBr$ and 0.05 M sodium phosphate buffer at pH 7.3. The numbers on each spectra refer to the compounds in Table I. **1**, mesoheme dimethyl ester; **2**, chelated mesoheme; **8**, di-C_4-chelated mesoheme; **10**, phenyl forked heme; **11**, forked heme; **12**, diforked heme. The upper set of curves are on a 0-0.1 absorbance scale and the lower, 0-1 scale.

and **8**-CO. The binding of the imidazole is increased by more than 10^3 when CO is in the sixth position.[14]

The reason that the introduction of strain into chelated hemes causes a change in dissociation mechanism rather than simply increasing the CO off rate, l, can be understood in terms of the relative CO off rates from five- and six-coordinated hemes. Deuteroheme-CO with no sixth ligand can be estimated to dissociate with a rate constant of 10^4 s^{-1} in benzene.[9h] This compares to about 0.03 s^{-1} for **3**-CO in toluene. Therefore k/k' of eq 15 need only be ~10^{-5} to lead to predominant base

$$\text{B—HmCO} \underset{k'}{\overset{k}{\rightleftharpoons}} \text{HmCO} \xrightarrow{4000\ s^{-1}} \text{Hm} \xrightarrow{\text{fast}} \text{HmB} \quad (15)$$

$$l = 0.03\ s^{-1} \quad (16)$$

elimination. The introduction of strain seems to increase the magnitude of k or k/k' faster than it increases l and this leads to the switchover to base elimination. Direct dissociation of dioxygen appears to be more sensitive to strain and direct dissociation is faster with dioxygen than with CO. Therefore it competes successfully with loss of base and such change in the dioxygen dissociation mechanism does not occur in the systems we have studied so far. These forked hemes have therefore been used as models for the behavior of T-state hemoglobin toward dioxygen.[9c]

Without the present evidence for a change in the mechanism of carbon monoxide dissociation we might have also offered the increased rate of CO dissociation (0.05 s^{-1} unstrained **2**-CO to 0.28 s^{-1} for strained **10**-CO) as a model for R- vs. T-state hemoglobin behavior. However, the tendency toward change in mechanisms with increasing strain means that the data on this particular series afford no definitive information concerning the R and T states of hemoglobin.

We *have* been able to devise kinetic models for R- and T-state hemoglobin reactions with carbon monoxide by use of a different stratagem.[9h] Extrapolation of the rates of reaction of CO with mixtures of heme and either 1-methylimidazole or 2-methylimidazole to infinitely high imidazole concentration

afforded the kinetics of the direct dissociation mechanism in these systems and the kinetic behavior of R- and T-state hemoglobins was duplicated rather well. We concluded that the change from R to T state accrues principally from the proximal base-heme strain and does not require the steric interference of distal side amino acid residues which seems to occur in other hemoproteins. The present results agree with this interpretation which is discussed in detail elsewhere.[9h]

Conclusions

Studies of carbon monoxide dissociation from a series of chelated heme compounds show that changes in heme side chain, proximal base electron donation, and solvent polarity do not affect the dissociation rates. By varying the number of imidazole arms attached to the heme-CO complexes we have shown that unstrained chelated heme-CO complexes react by direct dissociation and strained chelated heme-CO complexes by base elimination. These results corroborate our previous mechanistic conclusions based upon CO combination rates and they are also consistent with the postulate that hemoglobin cooperativity is governed by proximal base strain effects.

Experimental Section

Protohemin chloride (Sigma and Calbiochem), sodium dithionite (J. T. Baker), cetyltrimethylammonium bromide (Sigma Chemical Co.), myristyltrimethylammonium bromide (Aldrich Chemical Co.), reagent grade methanol and toluene (Mallinckrodt) and other solvents, and CO and O_2 gases (Matheson Gas Products) were used as received. Mesohemin and mesoporphyrin were prepared by standard literature preparations.[9c] Chromatography was performed on silica gel plates (Eastman no. 13L81), or on silica gel (Baker) columns. Deuterated solvents were obtained from Stohler Isotope Chemicals. All the heme compounds used in this work except **8** and **10** have been described elsewhere.[9e,g]

Nuclear magnetic resonance spectra were determined on a Varian HR-220 spectrometer equipped with Transform Technology TT-100 pulse/Fourier transform. Sweep widths of 4 kHz and 4K data points were used, and all shifts are reported downfield of internal Me_4Si.

Visible spectra were determined on a Cary 15 spectrophotometer.

Stopped-flow measurements were determined on an Aminco-Morro water-circulating copper heat sink was designed to fit under the observation chamber replacing the aluminum plate there. From this sink copper sheeting extends upward along two sides of the observation block to within 1 in. of the top of the chamber. A thermocouple was sealed onto the sink with the leads going out through the side port. The remainder of the chamber not in direct line of the monitoring beam was packed with cotton to reduce air currents. Water was circulated through the copper sink through tubing passing through the ports designed for air flow. Temperature was controlled to ±0.2 °C in both this sink and the heat sink around the plungers with a Haake Type F circulating pump. Temperature was monitored in the chamber using the thermocouple described above and around the plunger by a thermocouple sealed in a hole drilled in the plunger casing. Temperature was determined using a Leeds and Northrup Millivolt potentiometer 8960 and was found to be the same at both points within the accuracy of the potentiometer at all temperatures studied. Some of the rates were also determined with a standard Durrum stopped-flow apparatus.

Solutions for the trap method were prepared by adding the desired hemin to a tonometer containing solvent degassed by a stream of argon and pretreated with sodium dithionite. The hemin was dissolved in a small amount of solvent and added by syringe to the solution to give the desired concentration of reduced heme. Concentrations of the hemes were determined from the absorbance of the reduced hemes at their Soret maxima using their previously determined extinction coefficients.[9g] The diacetyl trap (**3**) (or potassium ferricyanide solution) was then transferred to the stopped-flow via syringe, being careful to minimize exposure of the solution to air. A carbon monoxide saturated solution was titrated into the other heme solution until it was converted to about 90–95% of its carbon monoxide saturated state. This solution was then transferred to the other port of the stopped-flow apparatus.

Rates were determined by following the transmittance change at or near the Soret maximum of the heme-CO complex (where maximum OD change was observed). The voltage output from the photomultiplier was recorded digitally through a microprocessor and rate constants were obtained as described elsewhere.[9h] Within a single run or between shots in a single pair of solutions, deviations in first-order rate constants were ±1%. Between complete runs, the rate variations were about ±10%. The acetyl heme (**3**) undergoes little or no absorbance change in this region in going from pentacoordinated to hexacoordinated heme. Photolysis was avoided by keeping the slit width at 0.5 mm in the Aminco instrument and 0.05–0.10 mm in the Durrum instrument.

The aqueous solvents used were prepared as follows: $MyrMe_3NBr$, 8 g, and $CetMe_3NBr$, 32 g, were dissolved and brought to 1 L with distilled water. A second solution which was 0.3 M in phosphate was prepared by dissolving 20.8 g of K_2HPO_4 and 4.5 g of KH_2PO_4 in distilled water and bringing the resulting solution to 500 mL. The pH was adjusted to 7.3 by small additions of H_3PO_4. The detergent solution and buffer solution were then mixed in equal volumes to give a final solution which was 0.15 M in phosphate buffered at pH 7.3 and contained 1.6% $CetMe_3NBr$ and 0.4% $MyrMe_3NBr$ by weight. The $CetMe_3NBr$–$MyrMe_3NBr$ mixture was used because pure $CetMe_3NBr$ often crystallizes from 2% solutions at about 22 °C.

Mesohemin Bis[4-(1-imidazolyl)butylamide] Chloride (8^+Cl^-).[19] To a stirred suspension of 50 mg (88 μmol) of mesoporphyrin and 5 drops of triethylamine was added dropwise 15 μL (176 μmol) of pivaloyl chloride in 10 mL of methylene chloride and the suspension stirred for 12 h under argon. The 1-(4-aminobutyl)imidazole[9e,19] (24.1 μL, 176.6 μmol) suspended in 10 mL of methylene chloride was added dropwise and stirring continued for 30 min. The solution was then washed with 1 N NaOH, then water, evaporated, and chromatographed on a silica gel column with methylene chloride–methanol–triethylamine (94.5:5:0.5) eluent. The infrared spectrum displayed a single carbonyl peak at 1650 cm^{-1}. NMR ($CDCl_3$): δ −3.87 (s, NH), 1.84 (t, 6 H), 3.03 (t, 4 H), 3.61, 3.59, 3.56 (3 s, 12 H), 4.03 (q, 4 H), 4.33 (t, 4 H), 6.38 (s, 2 H), 5.45 (d, 2 H), 6.32 (d, 2 H), 6.70 (d, 2 H), 10.02, 9.98, 9.95 (3 s, 4 H), assignments as in ref 9e.

Iron insertion was carried out as usual[9e] and the resulting hemin chloride chromatographed on a silica gel column with methylene chloride–methanol (4:1) containing 0.5% triethylamine, yield 22 mg of 8^+Cl^- (28% from mesoporphyrin).

3-(1′-Imidazolyl)propyl-3″-phenylpropylamine. Hydrocinnamaldehyde (Aldrich, 2.00 g, 14.9 mmol) and 3-(1′-imidazolyl)propylamine (2.02 g, 16.2 mmol) were dissolved in 200 mL of ethanol. Freshly prepared Raney nickel (W-2, ~1 g) was added as an ethanol suspension. The reaction mixture was placed in a Parr shaker and hydrogenated with 35 psi of hydrogen. The reaction was stopped when no further uptake of hydrogen was observed. The catalyst was filtered and the filtrate evaporated to a yellow oil. The crude amine was chromatographed on an alumina column (1 × 10 cm) eluting with chloroform–methanol (95:5 v/v). The first fraction was collected and rechromatographed on a silica gel column using the same eluent initially to remove impurities. Solvent was switched to chloroform–methanol (4:1 v/v) to elute pure amine, yield 0.58 g (16%). NMR ($CDCl_3$): δ 1.80 (m, 4 H), 2.63 (m, 6 H), 3.04 (s, 1 H), 3.95 (t, 2 H), 6.86 (d, 1 H), 6.99 (d, 1 H), 7.18 (m, 5 H), 7.42 (s, 1 H).

Mesohemin Mono[3-(1′-Imidazolyl)propyl-3″-phenylpropyl]amide Monomethyl Ester (10^+Cl^-). Mesoporphyrin mono[3-(1′-imidazolyl)propyl-3″-phenylpropyl]amide monomethyl ester was synthesized from mesoporphyrin monomethyl ester monoacid (200 mg, 340 μmol), pivaloyl chloride (60 μL, 500 μmol), and 3-(1′-imidazolyl)propyl-3″-phenylpropylamine (214 mg, 880 μmol) and purified by chromatography as described above, yield 152 mg (53%). NMR ($CDCl_3$): δ −3.85 (s, 2 H), 0.60 (m, 2 H), 1.46 (m, 2 H), 1.86 (t, 6 H), 1.99 (m, 2 H), 2.26 (q, 2 H), 2.59 (m, 2 H), 2.93 (m, 2 H), 3.12 (m, 2 H), 3.26 (m, 1 H), 3.38 (m, 1 H), 3.44–3.65 (m, 17 H), 4.07 (m, 4 H), 4.35 (m, 2 H), 4.99 (m, 1 H), 6.77 (m, 1 H), 6.99 (m, 3 H), 7.30 (m, 1 H), 9.91–10.08 (m, 4 H). Iron insertion was accomplished and purification carried out as described for 8^+Cl^-. The hemin was chromatographed and checked for purity in the usual manner, yield 80 mg (47%). The hemin displayed single spot purity on thin layer chromatography with different eluents.

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Preparation and Characterization of Beaded Poly(*N*-acrylylpyrrolidine): Bidirectional Synthesis of Cys-, His-, Gln-, or Glu-Containing Polypeptides[1]

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Abstract: The synthesis, characterization, and application of a poly(*N*-acrylylpyrrolidine) resin as an improved insoluble support for the preparation of peptides is described. The three-dimensional polymer was obtained by reverse-phase suspension copolymerization of the water-soluble monomers *N*-acrylylpyrrolidine, *N,N'*-bis(acrylyl)-1,2-diaminoethane, and *N*-acrylyl-1,6-diaminohexane hydrochloride to yield a resin in beads of predetermined size. This method of polymer preparation appears to be generally applicable for water-soluble monomers. The particular resin studied contained 4.4% cross-linking, 0.7 mmol of primary amine per g of polymer and was prepared with a monomer-to-solvent dilution ratio of 1:4. The resin has favorable swelling properties in solvents covering a broad range of polarities (CH_2Cl_2, alcohols, AcOH, DMF, and H_2O), which allow the adaptation of most of the techniques developed for the Merrifield method of solid-phase peptide synthesis as well as the use of procedures not previously compatible with polystyrene. The feasibility of amino acid and peptide attachment and detachment has been demonstrated with three types of polymer-to-peptide bridging groups: (1) a new *S*-carbamoyl group for the side-chain attachment of cysteine was used for a high yield bidirectional synthesis of deaminooxytocin, a potent biologically active analogue of oxytocin; (2) the dinitrophenylene group for the side-chain attachment of histidine was used for the bidirectional synthesis of thyrotropin-releasing hormone; (3) the benzyl ester group for COOH-terminal attachment and, moreover, for the side-chain attachment of glutamic acid. *N*-Benzyloxycarbonylprolylglutamyl-O^5-[4-(oxymethyl)phenylacetamidopoly(*N*-acrylylpyrrolidine)]glycinamide was assembled bidirectionally to demonstrate the feasibility of preparing both glutamic acid and glutamine-containing peptides from the same polymer-attached peptide intermediate; saponification and ammonolysis were the respective methods of choice for the liberation of the Z-Pro-Glu-Gly-NH_2 and Z-Pro-Gln-Gly-NH_2 model peptides. Reaction conditions used for detachment are nonacidic and different from those normally used for removal of N^{α}-amino group protection. Coupling reactions on the resin have been demonstrated in such diverse solvents as CH_2Cl_2, DMF, and aqueous media.

The introduction of the principle of peptide assembly on insoluble supports marked a milestone for the field of peptide synthesis.[2] When the term "solid-phase peptide synthesis" was originally introduced, it was generally felt that the polymer would act only as an inert, solid support. However, during the last decade extensive experience with polystyrene-based resin beads has shown that the insoluble support does actually have a dynamic influence on the synthesis of the peptide. Unfortunately, the influences on mass transport of reagents, solvation of polymers as well as peptide, and reaction rates of acylation as well as deprotection have been mostly negative because of physicochemical incompatibility of polystyrene with the attached peptide.[3,4] Emphasis to overcome these problems has focused on making the growing peptide more compatible with the polymer through the application and development of appropriate protecting groups.[5,6]

Instead of attempting to make the growing peptide chain conform to the requirements of a polystyrene-based resin, there appears to be a greater potential to improve the techniques of peptide synthesis by developing insoluble polymeric supports which are physicochemically compatible with the backbone structure of a peptide.[3,4] Ideally, a new polymer would influence the course of the synthetic reactions in a positive manner by providing an environment more favorable for the reactions.

Several proposals have been made for the development of insoluble polymeric supports with chemical structures resembling those of polypeptides. For example, Anfinsen[7] suggested the use of a polypeptide per se as a polymeric support. A cross-linked poly(L-lysine) has been reportedly used for oligonucleotide synthesis.[8] Inman and Dintzis proposed the use of cross-linked polyacrylamide for peptide synthesis,[9] but,

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