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Nitric Oxide and Carbon Monoxide Equilibria of Horse Myoglobin and (*N*-Methylimidazole)protoheme. Evidence for Steric Interaction with the Distal Residues†

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ABSTRACT: The Soret absorption maxima and extinction coefficients of the CO and NO complexes of horse myoglobin and (NMeIm)protoheme (NMeIm = 1-methylimidazole) have been determined. The partition coefficient N, equal to the ratio $P_{1/2}(CO)/P_{1/2}(NO)$, has been determined spectrophotometrically for horse myoglobin and (NMeIm)protoheme. $P_{1/2}$(NO) values calculated from the partition coefficients are 5.7×10^{-7} mmHg for (NMeIm)protoheme and 1.1×10^{-6} mmHg for horse myoglobin. The ratio of $P_{1/2}(NO)$ values for protein and model is 1.9 which is similar to a value of 1.6 reported for the ratio of $P_{1/2}(O_2)$ values. These values may be compared to a ratio of 15 for CO binding to protein and model complexes. This different ratio for CO provides further evidence for steric interaction of the bound CO with the protein based on a consideration of the preferred nonlinear geometry of Fe–NO and Fe–O_2 and the linear geometry of Fe–CO.

Data on infrared stretching frequencies have been extremely important in revealing the nature of protein ligand bonds. Over the last several years the literature has provided CO infrared stretching frequencies of various hemoproteins (Caughey, 1970; Caughey et al., 1969; Alben & Caughey, 1968). Many normal hemoglobins exhibit similar CO frequencies, but abnormal hemoglobins with the distal histidine replaced by amino acid residues bearing hydrophilic side chains have considerably higher CO frequencies (Caughey et al., 1969). The stretching frequencies of these mutant hemoglobins approach the ν_{CO} of model heme complexes (Collman et al., 1976).

Differences in the ν_{CO} have been interpreted in terms of the structure and ligand binding properties of hemoprotein and model complexes. The Fe–CO bond has been found to be linear in the nonhindered model systems Fe(TpivPP)(NMeIm)CO[1] (Hoard, 1975) and Fe(TPP)(py)CO (Peng & Ibers, 1976). X-ray data (Huber et al., 1970; Padlan & Love, 1974) indicate that in proteins the CO to Fe bond is bent or tilted. A neutron diffraction study has indicated a Fe–C–O bond angle of 135° in horse myoglobin (Norvell et al., 1975). These studies indicate that amino acid residues in the heme pocket are close enough to cause CO to bend or tilt from its preferred linear structure. Specifically, valine (residue E11) and the distal histidine (residue E7) are capable of interacting with CO when it is bound to horse myoglobin (Norvell et al., 1975). Caughey has attributed the lower ν_{CO} in normal hemoglobins and myoglobins to a donation of electron density from the nitrogen of the distal histidine to the partially electropositive carbon atom of the bent or tilted CO molecule (Caughey, 1970). Collman has suggested that the lower stretching frequencies result from steric hindrance which distorts the CO from its preferred linear structure (Collman et al., 1976).

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[1] Abbreviations used: NMeIm, 1-methylimidazole; PP, protoporphyrinate; TpivPP, *meso*-tetrakis($\alpha,\alpha,\alpha,\alpha$-*o*-pivalamidophenyl)porphyrinate; NTrIm, 1-tritylimidazole; py, pyridine; $P_{1/2}$, partial pressure of gas at half-saturation; Mb, myoglobin; Im, imidazole; TPP, *meso*-tetraphenylporphyrinate; CTAB, cetyltrimethylammonium bromide.

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In contrast, theoretical calculations using FePP and NMeIm show that oxygen should be bound in a bent geometry (Kirchner & Loew, 1977). X-ray structural analysis has shown this is to be the case in a nonrestricted model heme complex (Fe–O–O angle $\simeq$ 135°) (Collman et al., 1974; Jameson et al., 1978). It is well-known that bound O_2 in the hemoproteins is bent (Rodley & Robinson, 1972; Barlow et al., 1973; Phillips, 1978; Makinen et al., 1978). It has therefore been proposed that steric interactions decrease the CO affinity of hemoproteins, while not altering the O_2 affinity of hemoproteins (Caughey, 1970; Antonini & Brunori, 1971, pp 91–93; Heidner et al., 1976; Peng & Ibers, 1976; Collman et al., 1976). X-ray data indicate that the Fe–NO bond is also bent in model systems (Perry, 1968; Piciulo et al., 1974). Infrared frequencies (Maxwell & Caughey, 1976) and EPR spectra (Barlow & Erecinski, 1979) of hemoproteins are consistent with a nonlinear Fe–NO bond. If nitric oxide is bent with a bond angle similar to O_2, it should not be affected by steric hindrance in hemoproteins. CO, on the other hand, would prefer to bind in a linear geometry and would thus appear to experience significant steric hindrance in binding to the hemoproteins. It is our hypothesis that the ratio of the O_2 affinity of Mb to the O_2 affinity of a model, $P_{1/2}(O_2)_{Mb}/P_{1/2}(O_2)_{model}$, should be similar to the ratio of the NO affinity of Mb to the NO affinity of a model, $P_{1/2}(NO)_{Mb}/P_{1/2}(NO)_{model}$. A comparison of the affinities of the hemoproteins and the models, in terms of $P_{1/2}$ values, for CO, O_2, and NO may indicate the extent to which steric hindrance affects CO binding to proteins. The present work describes the measurement of the NO affinities of a (1-methylimidazole)protoheme complex and of horse myoglobin.

Materials and Methods

Horse heart myoglobin (Type III), crystalline bovine hemin (FePP) (Type I), and 1-methylimidazole (NMeIm) were purchased from Sigma Chemical Co. Ultra pure carbon monoxide, chemically pure nitric oxide, and a 403-ppm nitric oxide in nitrogen gas mixture were obtained from Matheson. All other chemicals used were of reagent grade. A Matheson Model 7372T gas proportioner with Kel-F packing equipped with No. 600 flowmeter tubes was used to prepare mixtures of CO and NO. The gas proportioner has a variable flow rate ranging from 4 to 57 cm^3/min for both carbon monoxide and nitric oxide in nitrogen as calibrated in our laboratory. Thunberg tubes with 19/22 ground glass fittings from Lab Glass were converted to thunberg cuvettes by replacing the lower half of the tube with square 1-cm i.d. glass tubing. A glass socket for a rubber septum was added on the top of the side arm to permit the introduction of gases. Absorption spectra were recorded on a Cary 14R spectrophotometer. Measurements of pH were made with a Radiometer PHM 64 pH meter.

Preparation of FePP(NMeIm)$_2$ Complexes. Hemin was dissolved in an aqueous solution containing 20% v/v NMeIm and the pH was adjusted to 9.0, yielding $Fe^{III}PP(NMeIm)_2$. This concentration of NMeIm was found necessary to give only the six-coordinate $Fe^{II}PP(NMeIm)NO$ complex in the presence of NO. At lower concentrations of NMeIm, there is evidence for the formation of the five-coordinate $Fe^{II}PPNO$ complex in the presence of NO in keeping with previous observations (Maxwell & Caughey, 1976). Deaeration of a solution of $Fe^{III}PP(NMeIm)_2$ with nitrogen for 1 hour, followed by addition of dithionite from the side arm, yielded $Fe^{II}PP(NMeIm)_2$.

Preparation of $Fe^{II}PP(NMeIm)CO$ and $Fe^{II}PP(NMeIm)NO$. In the preparation of the six-coordinate $Fe^{II}PP(NMeIm)CO$, oxygen was rigorously excluded. CO was passed over the aqueous solution of $Fe^{III}PP(NMeIm)_2$ for 2 h and subsequently bubbled through the solution for 30 min prior to the addition of 0.0005 g of ascorbate from the side arm. CO was then bubbled through the solution for 1 additional h, yielding $Fe^{II}PP(NMeIm)CO$.

For the preparation of $Fe^{II}PP(NMeIm)NO$, NO was bubbled through a solution of $Fe^{II}PP(NMeIm)CO$ until the complex was completely converted to the nitrosyl form as evidenced by UV–visible spectroscopy. We were unable to prepare $Fe^{III}PP(NMeIm)NO$; NO reduced $Fe^{III}PP(NMeIm)_2$ to $Fe^{II}PP(NMeIm)NO$ over a period of several hours without the apparent intermediate formation of the ferric nitric oxide complex.

Preparation of Carbonyl- and Nitrosylmyoglobin. For the preparation of carbonylmyoglobin, CO was passed over an aqueous solution of myoglobin containing 0.4 M phosphate buffer, pH 7.0, at 22 °C in the thunberg cuvette for 12 h after which 0.0005 g of ascorbate was added from the side arm. CO was then passed over the solution for an additional 12 h until pure carbonylmyoglobin was obtained. For the preparation of ferrous nitrosylmyoglobin, NO was bubbled through the solution of the carbonylmyoglobin until the carbonyl complex was completely converted to the nitrosyl form.

Preparation of Nitric Oxide Metmyoglobin. For the preparation of NO metmyoglobin, a solution of horse heart metmyoglobin in 0.4 M phosphate buffer, at pH 7.0, 22 °C, was deaerated with nitrogen and then bubbled rapidly with NO for 3 min to ensure complete complexation, and the spectrum was recorded immediately thereafter. The resulting solution gave a visible absorption spectrum that corresponded to the NO metmyoglobin complex reported by Wittenberg et al. (1967). Upon standing for 5 h, the solution showed spectral changes characteristic of the conversion of myoglobin from the Fe(III) form to the Fe(II) form. It has been reported that NO slowly reduces metmyoglobin (Drabkin & Austin, 1935). The same NO myoglobin spectrum was obtained by adding NO to carbonylmyoglobin.

Determination of NO Affinities. CO and 403-ppm NO in N_2 were mixed via the calibrated gas proportioner and the individual flows were adjusted appropriately to obtain the desired ratios of P(CO) and P(NO). The gas mixture was bubbled through a solvent saturation tower containing either water (for myoglobin solutions) or 20% NMeIm in water, to eliminate sample concentration changes due to solvent evaporation upon bubbling. The solvent-saturated gas mixture was then bubbled into the solutions containing the heme carbonyls by using a 20-gauge stainless steel needle. Absorbance readings were taken after raising the needle above the solution.

Absorbances were monitored at 418 nm for the model heme complexes and at 424 nm for the myoglobin complexes. Sixty minutes of bubbling was generally required to obtain a constant absorbance. If after an additional 30 min of bubbling the absorbance had not changed, the system was considered to be at equilibrium. Bubbling occasionally resulted in the denaturation of some of the myoglobin. This could be prevented by lowering the flow rate and limiting the bubbling time. Only experiments which exhibited good isosbestic points were used for calculations.

Results

Absorption spectra were first measured for all possible species of model and hemoprotein complexes which are associated with the ligand binding equilibria in solution. Figure 1A shows the Soret region of the absorption spectra of $Fe^{III}PP(NMeIm)_2$, $Fe^{II}PP(NMeIm)_2$, $Fe^{II}PP(NMeIm)CO$,

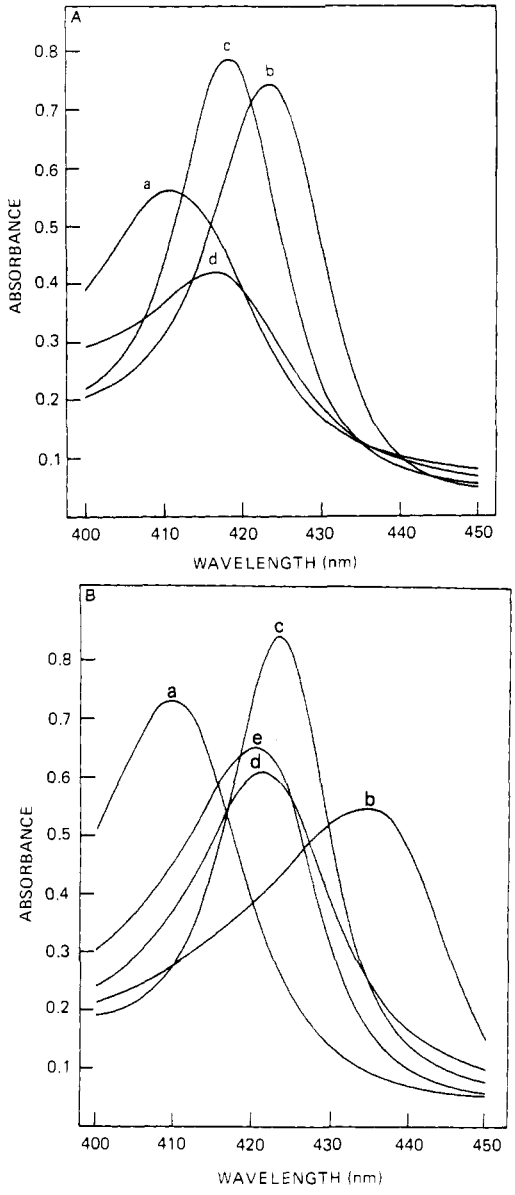

FIGURE 1: (A) Soret absorption spectra of protoheme complexes in 20% NMeIm, pH 9.0, 0.40 M phosphate buffer, and 22 °C. [Protoheme] = 3.6×10^{-6} M. (a) $Fe^{III}PP(NMeIm)_2$; (b) $Fe^{II}PP(NMeIm)_2$; (c) $Fe^{II}PP(NMeIm)CO$; (d) $Fe^{II}PP(NMeIm)NO$. (B) Soret absorption spectra for horse myoglobin, pH 7.0, 0.40 M phosphate buffer, and 22 °C. [Myoglobin] = 4.1×10^{-6} M. (a) Metmyoglobin; (b) myoglobin; (c) CO myoglobin; (d) NO myoglobin; (e) NO metmyoglobin.

and $Fe^{II}PP(NMeIm)NO$. Corresponding absorption maxima are recorded in Table I.

Figure 1B shows the Soret region of the absorption spectra of $Fe^{III}Mb$, $Fe^{II}Mb$, $Fe^{II}MbCO$, $Fe^{II}MbNO$, and $Fe^{III}MbNO$. By use of an extinction coefficient of 121 mM^{-1} cm^{-1} for oxymyoglobin, the extinction coefficients were calculated for $Fe^{III}NO$ myoglobin and $Fe^{II}NO$ myoglobin. The corresponding absorption maxima and extinction coefficients are recorded in Table I for comparison to those of the model heme complexes. Absorption maxima and relative extinction coefficients for $Fe^{II}Mb$, $Fe^{III}Mb$ and carbonylmyoglobin are in good agreement with those previously reported (Antonini & Brunori, 1971, p 19).

The differences between the ferrous carbonyl and nitrosyl compounds were much greater in the Soret region than in the visible region; for this reason the Soret region was chosen to follow absorption changes in the equilibration of the carbonyl

Table I: UV-Visible Absorption Maxima and Extinction Coefficients for (NMeIm)protoheme and Myoglobin Complexes

	(NMeIm)protoheme		myoglobin	
form	λ_{max} (nm)	ϵ (mM^{-1} cm^{-1})[a]	λ_{max} (nm)	ϵ (mM^{-1} cm^{-1})[b]
Fe(III)	411.5[c]	156	409.5	186
Fe(II)	424.0[c]	210	435.0	121
$Fe^{II}CO$	419.0	219	423.5	207
$Fe^{II}NO$	415.0	126	421.5	147
$Fe^{III}NO$	none		420.5	155

[a] Extinction coefficients were based on the concentration of heme determined by the pyridine hemochrome method described by Falk (1964) using an $\epsilon = 191.5$ mM^{-1} cm^{-1}. [b] Extinction coefficients were calculated by using an $\epsilon = 121$ mM^{-1} cm^{-1} for deoxymyoglobin (Antonini & Brunori, 1971, p 19). [c] Fe(PP)-$(NMeIm)_2$.

Table II: Binding of NO to (NMeIm)protoheme at Different Partial Pressures of CO and NO[a]

$P(CO)/P(NO)$[b]	% nitrosyl complex	N[c]
4050	33.3	2030
3810	35.9	2130
2250	48.7	2130
1330	60.9	2070
750	73.4	2060
225	90.0	2030

[a] At 22 °C in 20% v/v NMeIm at pH 9.0. [b] The partial pressure of CO divided by the partial pressure of NO. [c] N is defined as $[Fe^{II}PP(NMeIm)NO]P(CO)/[[Fe^{II}PP(NMeIm)CO]P(NO)]$.

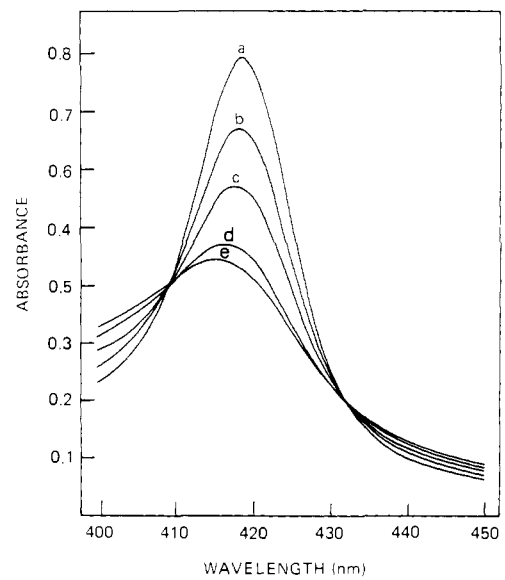

FIGURE 2: Equilibration of $Fe^{II}PP(NMeIm)CO$ with different ratios of carbon monoxide to nitric oxide, pH 9.0, 0.40 M phosphate buffer, and 22 °C. [Protoheme] = 3.6×10^{-6} M. (a) $Fe^{II}PP(NMeIm)CO$; (b) $P(CO)/P(NO) = 4050$; (c) $P(CO)/P(NO) = 1300$; (d) $P(CO)/P(NO) = 225$; (e) $Fe^{II}PP(NMeIm)NO$.

compounds with NO. The following are the equilibria of interest:

$$Fe^{II}PP(NMeIm)CO + NO \rightleftharpoons Fe^{II}PP(NMeIm)NO + CO$$

$$Fe^{II}MbCO + NO \rightleftharpoons Fe^{II}MbNO + CO$$

Figures 2 and 3 show the absorption changes associated with varying pressures of NO and CO. As the ratio of NO to CO was increased, the percentage of nitrosyl complex increased

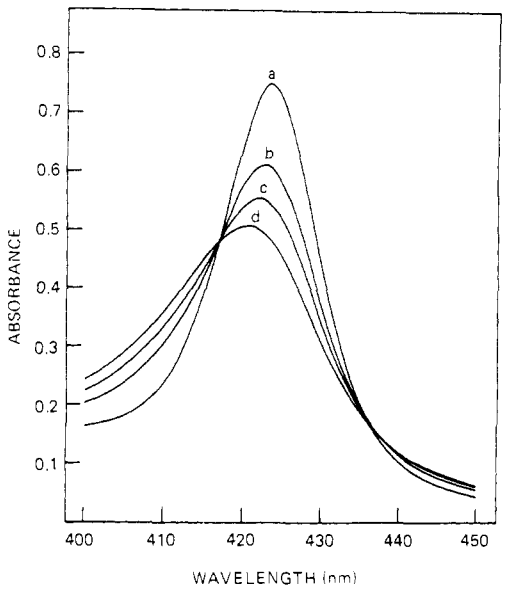

FIGURE 3: Equilibration of horse carbonylmyoglobin with different ratios of carbon monoxide to nitric oxide, pH 7.0, 0.40 M phosphate buffer, and 22 °C. [Myoglobin] = 3.6×10^{-6} M. (a) Carbonylmyoglobin; (b) $P(CO)/P(NO) = 8080$; (c) $P(CO)/P(NO) = 4000$; (d) nitrosylmyoglobin.

Table III: Binding of NO to Horse Myoglobin at Different Partial Pressures of CO and NO[a]

$P(CO)/P(NO)$	% nitrosyl complex	N[b]
32 300	32.0	15 200
29 000	35.7	16 100
19 000	44.3	15 100
16 700	48.6	15 800
8 080	66.2	15 900
4 960	77.7	17 400
3 450	83.0	16 800

[a] At 22 °C and pH 7.0. [b] N is defined as $[MbNO]P(CO)/[[MbCO]P(NO)]$.

as shown in Tables II and III. The partition coefficient between NO and CO, N, was calculated from the equation

$$N = \frac{[Fe^{II}NO]}{[Fe^{II}CO]} \frac{P(CO)}{P(NO)}$$

where $P(CO)$ and $P(NO)$ represent the partial pressures of CO and NO. The N values are constant for different ratios of NO and CO and give an average N of 16 000 ± 1000 for myoglobin and 2100 ± 50 for (NMeIm)Fe^{II}PP. Consider a gas mixture which results in a solution containing 50% nitrosyl complex and 50% carbonyl complex. By definition, the pressure of CO corresponds to $P_{1/2}(CO)$ and the pressure of NO corresponds to $P_{1/2}(NO)$; thus, from the previous equation $N = P_{1/2}(CO)/P_{1/2}(NO)$.

The experimental data for the equilibration of Fe^{II}PP(NMeIm)CO with NO give the ratio of ${}^6P_{1/2}(CO)/{}^6P_{1/2}(NO)$, where ${}^6P_{1/2}(CO)$ and ${}^6P_{1/2}(NO)$ are the pressures for half-complexation of CO and NO to the six-coordinate model, $Fe^{II}PP(NMeIm)_2$. The following binding scheme was suggested by Rougée & Brault (1975), where H represents heme and L is NMeIm:

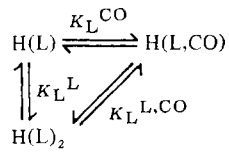

Table IV: Comparison of $P_{1/2}$[a] Values in Millimeters of Mercury

compd	$P_{1/2}$ (mmHg) O_2	CO	NO
model	0.45[b]	0.0012[b]	5.7×10^{-7}[c]
horse Mb	0.70[d]	0.018[e]	1.1×10^{-6}
horse Mb/model	1.6[f]	15[f]	1.9[f]

[a] $P_{1/2}$ is the partial pressure of a gas when a compound is half-complexed. [b] Mesoheme mono-4-(1-imidazolyl)butylamide monomethyl ester (Sharma et al., 1978; Geibel et al., 1978). [c] Fe^{II}PP(NMeIm)NO. [d] Antonini & Brunori (1971, p 221). [e] Antonini & Brunori (1971, p 224). [f] Ratio of the $P_{1/2}$ value of myoglobin to the $P_{1/2}$ value for the model.

A corresponding scheme may be used for NO equilibria. Expressed in terms of these equilibrium constants, the equilibrations provide a measure of $K_L^{L,NO}/K_L^{L,CO}$. $K_L^{L,NO}/K_L^{L,CO}$ can be obtained directly from the ratio of ${}^6P_{1/2}$ values as given by

$$\frac{{}^6P_{1/2}(CO)}{{}^6P_{1/2}(NO)} = \frac{K_L^{L,NO}s_{NO}}{K_L^{L,CO}s_{CO}}$$

where s_{NO} and s_{CO} are the solubilities of NO and CO, respectively. The ratio ${}^5P_{1/2}(CO)/{}^5P_{1/2}(NO)$ for the five-coordinate species Fe^{II}PP(NMeIm) is required for comparison to myoglobin. This ratio is given by

$$\frac{{}^5P_{1/2}(CO)}{{}^5P_{1/2}(NO)} = \frac{K_L^{NO}s_{NO}}{K_L^{CO}s_{CO}}$$

The relationships

$$K_L^{CO} = K_L^{L}K_L^{L,CO}$$

$$K_L^{NO} = K_L^{L}K_L^{L,NO}$$

$$K_L^{L,CO}/K_L^{L,NO} = K_L^{CO}/K_L^{NO}$$

indicate that

$$\frac{{}^6P_{1/2}(CO)}{{}^6P_{1/2}(NO)} = \frac{{}^5P_{1/2}(CO)}{{}^5P_{1/2}(NO)}$$

Equilibrium constants have been measured for the binding of O_2 and CO to the nonhindered model mesoheme mono-4-(1-imidazolyl)butylamide monomethyl ester (Sharma et al., 1978; Geibel et al., 1978). The corresponding $P_{1/2}$ values for O_2 and CO were calculated from these equilibrium constants and the solubilities of O_2 and CO in water. The $P_{1/2}(NO)$ was calculated from the $P_{1/2}(CO)$ and the experimentally determined value for N. Likewise, the $P_{1/2}(NO)$ for horse myoglobin was calculated from a value of $P_{1/2}(CO)$ (Antonini & Brunori, 1971, p 224) and the experimentally determined value for N. $P_{1/2}$ values for the model systems and horse myoglobin are recorded in Table IV. Ratios of $P_{1/2}$ values of protein and model complexes for each of the gaseous ligands are listed in row three of Table IV.

Discussion

The model and hemoprotein complexes described in Figure 1 and Table I exhibit similar spectral characteristics. In both the model and the protein, the Soret absorption maxima of the NO complexes are blue-shifted and have smaller extinction coefficients relative to the CO complexes. While the CO complexes of the model and protein exhibit narrow bandwidths, the absorptions of the NO complexes are considerably broader. The $Fe^{II}CO$ and $Fe^{II}NO$ Soret absorption maxima for the model are blue-shifted relative to the corresponding spectra of myoglobin. This shift may be associated with differences between the heme environment in the protein and in the model.

It is noteworthy that a NO complex was not observed for the Fe(III) model while it could be observed for the protein. This difference may be attributed to the smaller apparent affinity of the Fe(III) state for NO relative to the Fe(II) state as observed for myoglobin in addition to the competition of the excess NMeIm for the sixth coordination position.

The equilibrations provide $P_{1/2}$(NO) for protein and model complexes. The $P_{1/2}$(NO) values differ from one another by only a factor of 1.9. This ratio may be compared to ratios of 1.6 for O_2 and 15 for CO. The results show similar ratios for O_2 and NO binding.

The $P_{1/2}$(NO) values suggest that NO affinity of hemoproteins is not reduced relative to the models. This conclusion is consistent with observations which indicate that the Fe–NO bond is bent in models and proteins similar to the Fe–O_2 bond.

X-ray data on the non sterically hindered models FeBrNO-$(das)_2^+$ [das = *o*-phenylenebis(dimethylarsine)] (Perry, 1968) and Fe(TPP)(Im)(NO) (Piciulo et al., 1974) have indicated Fe–NO bond angles of 148 and 142°, respectively. The IR stretching frequencies of Fe^{II}PP(NMeIm)NO and hemoglobin A, 1618 and 1615 cm^{-1}, respectively, are both reported to be consistent with a bent Fe–NO bond (Maxwell & Caughey, 1976). Recently, EPR spectra of nitrosylcytochrome *c* oxidase have been measured and suggest a Fe–NO bond angle of 135° (Barlow & Erecinska, 1979). It has been suggested that NO complexes of Fe^{II}PP are stabilized by bending of the Fe–NO bond due to a lowering of energy of the one electron in an antibonding π^* orbital (Enemark & Feltham, 1974).

Likewise, X-ray structural data indicate that the Fe–O_2 bond in both models and proteins is bent. Recent X-ray evidence on oxymyoglobin (Phillips, 1978; Makinen et al., 1978) shows a bent Fe–O–O bond as proposed by Pauling (1964). The oxyheme model complexes Fe(TpivPP)(NTrIm)O_2 and Fe(TpivPP)(NMeIm)O_2 have been found to exhibit similar stretching frequencies (Collman et al., 1976). Crystallographic analysis of Fe(TpivPP)(NMeIm)O_2 indicates that the Fe–O_2 angle is approximately 135° (Collman et al., 1974; Jameson et al., 1978).

The ratios of 1.9 for $P_{1/2}(NO)_{horse\text{-}Mb}/P_{1/2}(NO)_{model}$ and 1.6 for $P_{1/2}(O_2)_{horse\text{-}Mb}/P_{1/2}(O_2)_{model}$ are therefore consistent with our expectation that NO and O_2 binding should be similar if the preferred bond angles are equivalent in both the models and the proteins.

In contrast, $P_{1/2}$(CO) is 15 times greater for the protein than for the model. This may be understood in terms of the observed differences between Fe–CO bond angles in the models and those in the proteins. X-ray structural data on the non-hindered models Fe(TpivPP)(NMeIm)CO (Hoard, 1975) and Fe(TPP)(py)CO (Peng & Ibers, 1976) indicate that the Fe–CO bond is linear. In comparison, there is abundant evidence that the Fe–CO bond is not linear in hemoproteins. Neutron diffraction analysis of horse myoglobin indicates that the CO oxygen is 0.8 Å below the axis linking the heme-linked histidine with the iron atom, resulting in a Fe–CO bond angle of 135° (Norvell et al., 1975). It was observed that the distal residues histidine-E7 and valine-E11 are within van der Waals distance of the bound CO. Carbon monoxide has also been found to be inclined to the heme plane in insect erythrocruorin (Huber et al., 1970), horse hemoglobin (Heidner et al., 1976), and bloodworm hemoglobin (Padlan & Love, 1974). It has been suggested that the bent bond in carbonylerythrocruorin may be due to steric restrictions imposed by isoleucine-E11. In horse hemoglobin, steric interactions have also been indicated between CO and the side chains of histidine-E7 and valine-E11. In comparison to the ratios of 1.6 and 1.9 for O_2 and NO given in Table IV, the ratio of 15 for CO is thus supportive evidence of a nonbonding steric interaction.

It should be pointed out that the five-coordinate models described by Traylor and collaborators (Geibel et al., 1978) contain mesoheme, while our model and myoglobin contain protoheme. The $P_{1/2}$ values for the mesoheme complexes may be slightly higher than the $P_{1/2}$ value for a protoheme complex (Antonini & Brunori, 1971, pp 287 and 229) which may affect the calculated $P_{1/2}$(NO) value for our model but not the relative affinities for CO and NO. In addition, studies on reconstituted myoglobin and hemoglobin indicate that the relative binding of O_2 and CO does not change with different hemes (Antonini & Brunori, 1971, pp 287 and 229). Thus, our relative binding constants are subject to only minimum error. It may also be noted that the $P_{1/2}$(CO) and $P_{1/2}(O_2)$ values calculated from the on/off kinetic data and the solubilities in water are subject to some uncertainty because the solubility of CO and O_2 in cetyltrimethylammonium bromide (CTAB) micelles may be different from the solubilities in water. However, the relative solubilities of O_2 and CO in different solvents are sufficiently similar that the ratios of $P_{1/2}$ values of myoglobin to the $P_{1/2}$ values for the model should be in the proportion 1.6:15:1.9.

Attempts have been made to correlate infrared frequencies to the strength of the Fe–CO bond (Barlow et al., 1973), to the photodissociation quantum yields (Shimada et al., 1979), and to the CO affinity (Collman et al., 1976). The latter correlations have been attributed to the degree of steric hindrance by residues in the heme pocket on the bound CO molecule. Normal hemoglobins have a ν_{CO} of approximately 1951 cm^{-1} (Maxwell et al., 1974); myoglobins have a ν_{CO} of approximately 1945 cm^{-1} (Barlow et al., 1973). Denaturated hemoglobin (Wang et al., 1958) and many unhindered models (Collman et al., 1976) exhibit a ν_{CO} of 1970 cm^{-1}. Hemoglobin Zurich, with arginine substituted for histidine-E7, shows a ν_{CO} of 1958 cm^{-1} for the abnormal β chains and 1951 cm^{-1} for the normal α chains (Caughey et al., 1969). It was shown that the abnormal β chains bind CO with 3 times the affinity of the normal α chains (Wallace et al., 1976). Likewise, a lower stretching frequency has been associated with a higher $P_{1/2}$-(CO) value for the normal hemoproteins and models.

A ratio of $P_{1/2}$(CO) value of about 100 has earlier been noted (Collman et al., 1976) for the binding of CO to myoglobin and Fe^{II}PP(Im) in benzene. The $P_{1/2}$(CO) ratio of 15 reported in this paper is based on a comparison of CO binding to myoglobin and the binding constant for the unhindered mesoheme mono-4-(1-imidazolyl)butylamide monomethyl ester in aqueous 2% CTAB, as calculated from recent on/off kinetic data (Sharma et al., 1978; Geibel et al., 1978). The difference between these ratios may be due to either the solvent or to some property of the specific model used. In either case the comparisons indicate that CO is sterically hindered in proteins as shown by the binding data, although the latter comparison suggests that the degree of steric hindrance, while appreciable, may not be as great as originally indicated.

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Reversible Reductive Alkylation of Amino Groups in Proteins†

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ABSTRACT: Amino groups of proteins can be alkylated by treatment with a carbonyl compound and a reducing agent [Means, G. E., & Feeney, R. E. (1968) *Biochemistry 7*, 2192]. We are now reporting on a reversible method of reductive alkylation in which the amino groups are first alkylated by treatment with an α-hydroxy aldehyde or ketone in the presence of sodium borohydride. Since the chemical grouping $RNHCH_2C(OH)<$ (R = alkyl) is readily attacked by periodate to give the primary amine (RNH_2), this modification is effectively reversed by treatment with periodate. Reversal of the modification is effected by treating the modified protein with relatively low levels of $NaIO_4$ (10–20 mM) for ~30 min and is accompanied by a full or partial recovery of the activity lost as a result of modification. The best results were obtained with glycolaldehyde (HOH_2CCHO) and with acetol (HOH_2CCOCH_3). Glycolaldehyde reacts readily with amino groups to give modification at a high level but has the disadvantage that a second mole of the aldehyde can add relatively easily to the amino groups, giving a tertiary amine which is resistant to attack by periodate. With sodium borohydride as the reducing agent, ~20% of the amino groups modified by extensive treatment are converted to this irreversibly modified form. Higher levels of dialkylation are observed when sodium cyanoborohydride is used. Acetol, a ketone, is less reactive; with sodium borohydride as the reducing agent, only one molecule reacts with each amino group. Again, sodium cyanoborohydride can cause significant irreversible modification by favoring dialkylation of amino groups. The method was applied to four selected proteins: lysozyme, which is known to be rapidly inactivated by periodate; turkey ovomucoid, which is a trypsin inhibitor with an essential lysine residue and contains 25% carbohydrate; chicken ovomucoid, which is very similar in general structure to the turkey ovomucoid, but which has an essential arginine residue instead of a lysine; ribonuclease, in which the amino groups are essential for activity.

Reductive alkylation is a selective and versatile technique for the chemical modification of amino groups in proteins (Means & Feeney, 1968). It is accomplished by treating the protein with low concentrations of a simple aliphatic aldehyde or ketone and a reducing agent, usually sodium borohydride or sodium cyanoborohydride. The positive charge of the amino groups is retained, and the amounts of reducing agent used appear to be insufficient to cause reductive cleavage of disulfide bonds (Means, 1977).

We are now extending the earlier work in our laboratory

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