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Chemistry

Nature of O_2 and CO binding to metalloporphyrins and heme proteins

(hemoglobin/infrared vibrational spectra/metal bound superoxides/distal binding site)

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ABSTRACT **The O_2 vibration of dioxygen adducts of Fe and Co model complexes of $\alpha,\alpha,\alpha,\alpha$-tetrapivalamidophenylporphyrin ("picket fence" porphyrin, TpivPP) with 1-methylimidazole and 1-tritylimidazole as axial bases are reported, obtained with difference techniques between $^{16}O_2$, $^{18}O_2$, ^{16}O–^{18}O, and NO with a Fourier transform infrared spectrometer. Assignments of ν_{O_2} are (O_2)Fe(TpivPP) 1-methylimidazole, 1159 cm^{-1} in Nujol; (O_2)Fe(TpivPP) 1-tritylimidazole, 1163 in benzene; (O_2)Co(TpivPP) 1-methylimidazole, 1150 in Nujol; (O_2)Co(TpivPP) 1-tritylimidazole, 1153 in benzene. Comparisons with other known Fe, Co, Cr, and Ti dioxygen complexes are made, and it is concluded that the bent dioxygen ligand is best viewed as bound superoxide, O_2^-.**

The CO affinities of various hemoproteins and model systems are discussed. A correlation between the CO stretching frequency and its binding constant is described. The drastically lowered affinity of hemoproteins for CO compared with unencumbered models is attributed to steric hindrance in the distal binding site, which allows discrimination between the already bent Fe^{III}–O_2^- and the normally linear Fe^{II}–CO systems. If the affinity of hemoproteins in living systems for CO relative to O_2 were not decreased, then massive poisoning would result from endogenous CO.

A continuing controversy exists over the nature of ligand bonding to hemoproteins and metalloporphyrins. While it has been well established that both iron and cobalt bind dioxygen in a bent end-on fashion (1, 2), physical measurements (3–6) and theoretical calculations (7–10) have been used to support both the spin-paired dioxygen (M^{II}–O_2) (11) and the electron transfer superoxide (M^{III}–O_2^-) (12) models. Here we present infrared data on dioxygen "picket-fence" porphyrin complexes of iron and cobalt. These results strongly support the M^{III}–O_2^- formalism. The present data correct the preliminary report of an oxygen vibration at 1385 cm^{-1} (3, 13) which has since proved to be an artifact* (14). We are unaware of any previous reports of ν_{O_2} in model Fe and Co porphyrins. In addition, we discuss the nature of CO binding to ferrous porphyrins and the implication this has for the role of the apoprotein in biological oxygen carriers. It is our thesis that the distal binding site in hemoproteins functions to lower the carbon monoxide affinity.

MATERIALS AND METHODS

Benzene (spectroquality, Baker "Photrex") solutions of the "picket fence" porphyrin complexes (3, 14), Fe^{II}meso-tetra($\alpha,\alpha,\alpha,\alpha$-*o*-pivalamidophenyl)porphyrinato (TpivPP) and Co^{II} ($\alpha,\alpha,\alpha,\alpha$-TpivPP), 17 mM and 19 mM containing 5 and 2 equivalents of 1-tritylimidazole (NTrIm), respectively, were prepared under inert atmosphere. Separate samples of these solutions were exposed to an atmosphere of CO, NO, O_2, and >200 torr $^{18}O_2$ (both 54% and 90% enriched)†. Infrared spectral data of all solutions were collected between 2000 cm^{-1} and 750 cm^{-1} on a Digilab FTS 15 B Fourier transform spectrometer, at 1 cm^{-1} resolution. A RRIC XL0 variable pathlength cell fitted with NaCl windows and set to 0.1 mm was used. Difference spectra were computed using the standard software of the spectrometer. (These difference spectra are analogous to sample minus reference absorbance spectra on a conventional dual beam spectrometer.) The coefficients in the difference spectra were chosen to reduce to baseline the porphyrin bands at 1695 cm^{-1} and 1001 cm^{-1}; small amounts of a neat benzene spectrum were then added or subtracted, to account for slight changes in concentration and pathlength, so as to reduce to baseline the benzene bands at 1961 cm^{-1} and 1819 cm^{-1}. In addition, solid samples of Fe(TpivPP)(NMeIm) and Co(TpivPP)(NMeIm) exposed to O_2, $^{18}O_2$ (90% enriched), and NO were prepared as Nujol mulls between KBr plates; difference spectra were computed as above at 4 cm^{-1} resolution.

RESULTS

The spectrum of Fe(TpivPP)(NTrIm)O_2 in benzene versus benzene is shown in Fig. 1. The gaps left at 1480–1460 cm^{-1} and 1040–1020 cm^{-1} are due to the most intense bands of benzene whose absorbances in these regions exceed 99.9% under these conditions. The spectrum in benzene, apart from increased resolution, does not differ from those taken in KBr pellets and Nujol mulls.

Difference spectra of the Fe(TpivPP)(NTrIm) adducts of $^{16}O_2$ versus $^{18}O_2$ (both 90% and 54% enriched) are shown in Fig. 2. Similar spectra for the Co analog are shown in Fig. 3. The bands due to $^{16}O_2$, ^{16}O–^{18}O, and $^{18}O_2$ are evident, as is an unassigned feature in the region of 1110–1095 cm^{-1}. In the O_2 adduct versus NO adduct spectra, one observes the complete loss of the 1163 cm^{-1} peak in Fe and the 1153 cm^{-1} peak in Co and the appearance of new bands at 1080 cm^{-1} and 1077 cm^{-1}, respectively, upon going to $^{18}O_2$ (90%) which confirms the assignment of these bands. The shifts in stretching frequencies of $^{16}O_2$ and ^{16}O–^{18}O adducts of both Fe and Co match the harmonic oscillator prediction. However, the band due to $^{18}O_2$ is shifted to a considerably lower frequency than predicted. This, in conjunction with the unassigned feature in the 1100 cm^{-1} region, both in benzene solution and in Nujol mull, indicates that Fermi coupling, i.e., the splitting of an accidental

Abbreviations: TpivPP, *meso*-tetra($\alpha,\alpha,\alpha,\alpha$-*o*-pivalamidophenyl) porphyrinato; NTrIm, 1-tritylimidazole; NMeIm, 1-methylimidazole; $P_{1/2}$, partial pressure of gas at half-saturation; py, pyridine; one torr = 1.33×10^2 pascal.

* This band at 1385 cm^{-1} is due to an impurity found in occasional KBr samples (R. R. Gagne, personal communication).

† Recent studies in this laboratory on the binding constants of O_2 with both Fe(TpivPP) 1-methylimidazole (NMeIm) and Co(TpivPP) (NMeIm) show that the partial pressure of O_2 at half saturation ($P_{1/2}{}^{O_2}$) = 0.31 torr and $P_{1/2}{}^{O_2}$ about 100 torr, respectively, at room temperature. While both Fe and Co complexes bind NO, only Fe binds CO as a six coordinate complex.

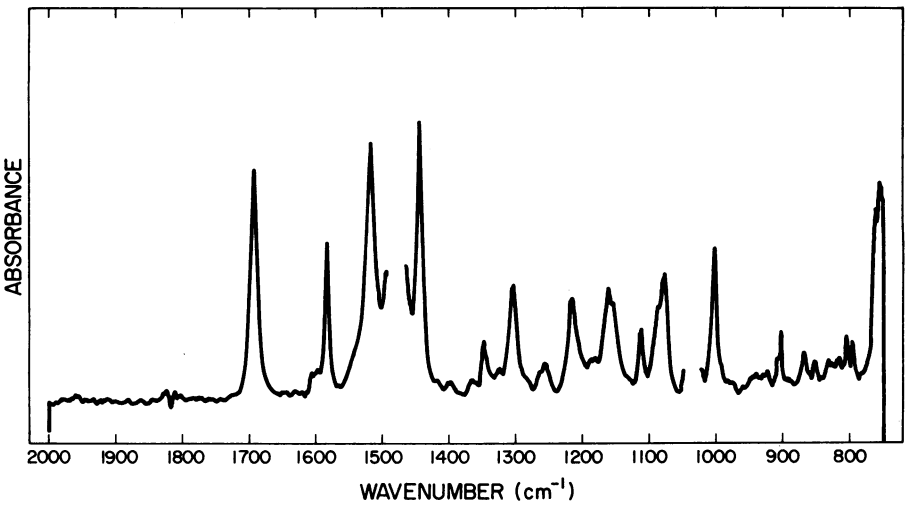

FIG. 1. Fe(TpivPP)(NTrIm)$^{16}O_2$ full spectra in benzene versus benzene.

degeneracy (15–18) has occurred between the strong porphyrin bands in this region and some of the isotopic O_2 stretches. The intensity of the observed $^{16}O_2$ bands decreases in the expected amount (to within experimental error) between the $^{16}O_2$ versus $^{18}O_2$ (90%) and the $^{16}O_2$ versus $^{18}O_2$ (54%) spectra. A more detailed analysis of these spectra is not possible at this time; however, the main points are clear. The O_2 stretch in both the Fe and the Co "picket-fence" porphyrin complexes is in the region of 1150 cm^{-1}, is relatively independent of the metal, and has been observed both in benzene solution and in Nujol mull.

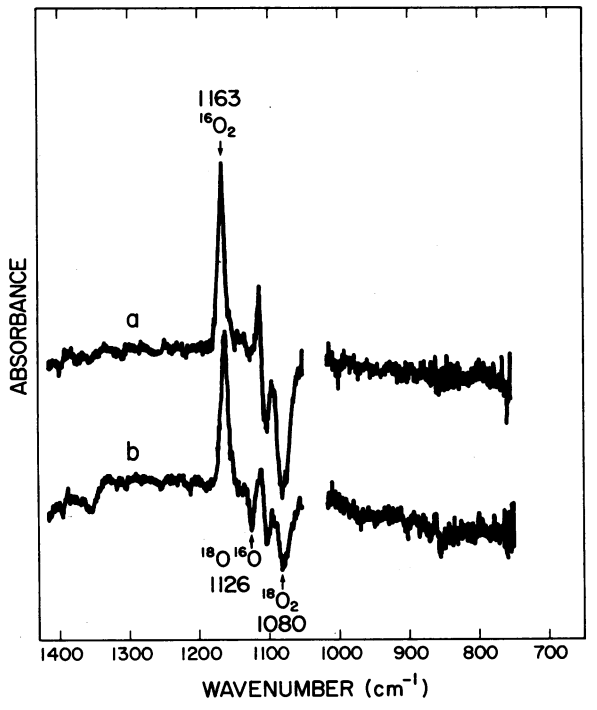

FIG. 2. Difference spectra of Fe(TpivPP)(NTrIm)$^{16}O_2$ minus Fe(TpivPP)(NTrIm)$^{18}O_2$. Spectrum *a* taken with 90% enriched $^{18}O_2$; spectrum *b* taken with 54% enriched $^{18}O_2$ in benzene solution. Extinction coefficient of $^{16}O_2$ band is 320 M^{-1} cm^{-1} with half height width of 10 cm^{-1} and an integrated intensity B = 3700 M^{-1} cm^{-2}. For comparison, the CO vibration in Fe(TpivPP)(NTrIm)CO has ϵ = 730 M^{-1} cm^{-1} and a half height width of 10 cm^{-1} and an integrated intensity B = 7500 M^{-1} cm^{-2}. Estimated errors in the intensity data are less than 20%. Gap left at 1020–1040 cm^{-1} due to intense benzene absorption.

Table 1 summarizes the observed band positions of the O_2 stretch, together with data from several other relevant systems. Table 2 presents the stretching frequencies and binding constants of the CO adducts of biological and model Fe porphyrins.

DISCUSSION

Nature of O_2 Binding. The frequencies of the O_2 vibration in our model porphyrins are close to those of ionic superoxides, whose range is about 1150–1100 cm^{-1} (29), with a gap of 200 cm^{-1} from the other dioxygen species, O_2 and $O_2^=$. The only

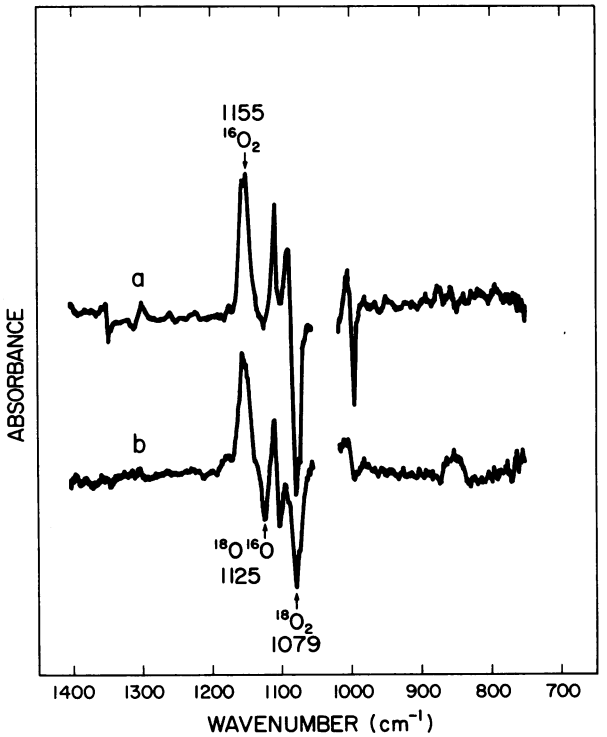

FIG. 3. Difference spectra of Co(TpivPP)(NTrIm)$^{16}O_2$ minus Co(FeTpivPP)(NTrIm)$^{18}O_2$. Spectrum *a* taken with 90% enriched $^{18}O_2$; spectrum *b* taken with 54% enriched $^{18}O_2$, in benzene solution. The bands attributed to the O_2 vibration are doublets resolved by 5 cm^{-1}: 1150 and 1155 cm^{-1} for $^{16}O_2$ and 1179 and 1174 cm^{-1} for $^{18}O_2$. Gap left at 1020–1040 cm^{-1} due to intense benzene absorption.

Table 1. Vibrational frequencies of dioxygen complexes

Compound	νO_2 (cm^{-1})	Ref.
Fe (TpivPP) (NTrIm)O_2*	1163 (1080, 1127†)	
Fe (TpivPP) (NMeIm)O_2‡	1159 (1075)	
MbO_2	1103 (1065)	18
HbO_2	1107 (1065)	19
Hb(deutFe)O_2	1106 (1065)	20
Co(TpivPP) (NTrIm)O_2*	1153§ (1077§, 1125)	
Co(TpivPP) (NMeIm)O_2‡	1150 (1077)	
Hb(deut Co)O_2	1105 (1065)	20
Co(acen)(py)O_2	~1130	21
Co(bzacen)(py)O_2	1128	22
$[Co(CN)_5O_2]^{-3}$	1138	23
Cr(TPP)(py)O_2	1142	24
Ti(OEP)O_2	898	25
$ML_2(CO)(X)O_2$¶	830–900	26
O_2^+	1860	17
O_2	1556	17, 27
O_2 ($^1\Delta$)	1484	17
O_2^-	1150–1100	17, 28, 29
$O_2^=$	740–850	17, 27, 29

Acen, *N,N'*-ethylenebis(acetylacetoniminato); bzacen, benzyl-*N,N'*-ethylenebis(acetylacetoniminato); py, pyridine; TPP, *meso*-tetraphenylporphyrinato; OEP, octaethylporphyrinato; deut, deuteroporphyrinato.

* Benzene solution.

† Wavenumber for $^{18}O_2$ and ^{16}O–^{18}O, respectively.

‡ Nujol mull.

§ These are, in fact, doublets with $\nu(^{16}O_2)$ = 1155, 1150 cm^{-1} and $\nu(^{18}O_2)$ = 1179, 1174 cm^{-1} due perhaps to the two different conformers of O_2 and NMeIm orientations.

¶ For example, M = Ir, Co, Os, Rh, Ni, Pt, Pd; L = triphenylphosphine, triphenylarsine, dialkylphenylphosphine; X = none, Cl, Br, I, N_3, NO_3.

other iron bound O_2 systems with which to compare are Hb and Mb; as we see in Table 1, all are in the spectral region associated with superoxide. However, the 50 cm^{-1} difference in the dioxygen vibration of the hemoproteins and the model [Fe(TpivPP)(NMeIm)O_2] is larger than would be expected on the basis of *electronic* differences between a tetraaryl and a natural porphyrin. For example, ν_{NO} for Fe(dimethyl protoporphyrinato)(NMeIm)NO is 1630 cm^{-1} in $CHCl_3$ or CH_2ClCH_2Cl (39), while for Fe(meso-tetra(pivalamidophenyl)porphyrinato)(NMeIm)NO is 1632 cm^{-1} in benzene or Nujol mull. In addition, ν_{CO} for Fe(dimethyl protoporphyrinato IX)(NMeIm)CO is 1970 cm^{-1} in KBr (37) and 1969 cm^{-1} in $CHCl_3$ (30, 39) and for Fe(TpivPP)(NMeIm)CO 1967 ± 2 cm^{-1} in benzene, Nujol, KBr, or CH_2Cl_2. One would expect, then, that the frequencies of the O_2 vibration would not change significantly in going from TpivPP to protoporphyrinato (i.e., the heme of Hb and Mb) *in the absence of* other differences in the local environment of the binding site such as steric interactions and local polarity. Because the actual normal mode which contains the O_2 stretch includes small amounts of other bends and stretches, small changes in geometry, as well as polarity, will cause shifts in the observed frequency, and the shift between the model complex and the hemoproteins, while interesting, is not surprising. The 1150 cm^{-1} band of the Co "picket fence" porphyrin complex is entirely consistent with the frequencies found for nonporphyrin mononuclear Co–O_2 adducts, as listed in Table 1. The small shift (10 cm^{-1}) between the ν_{O_2} in our Fe and Co complexes lends strong support to Maxwell and Caughey's surprising report of a shift of only 1 cm^{-1} between deuteroporphyrin Fe and Co reconstituted oxy-Hb.

In summary, the O_2 vibrational frequencies in our complexes are in the superoxide region. The influence of the metal is minimal. In conjunction with the electron spin resonance (6), x-ray photoelectron spectroscopy (5), and bond lengths from x-ray diffraction data (2, 41, 42), this and prior infrared evidence convincingly demonstrate that, for Co, 1:1 dioxygen adducts are best formulated as Co^{III}-O_2^-. It is now difficult to escape a similar conclusion for Fe and Cr (24). Thus, the best current picture is that the bent dioxygen ligand should be viewed as bound superoxide.

Table 2. Carbon monoxide adducts of Fe porphyrins

Compound	ν_{CO}(cm^{-1})	$P_{½}CO$*, 20°–25°, pH 7 (torr)	Ref.
MbCO†	1945	0.018	30, 31
HbCO†	1951	0.004 (0.035)‡	30, 31, 32, 33
HbCO, α or β chains		0.002	31
HbCO, insect (chironimus)	1959	0.002	34, 35
HbCO Zurich (β_{63} His → Arg)	1951, 1958	$P_{½}\beta < 1/3\ P_{½}\alpha$	36
Fe(P) (NMeIm) CO	1970§	0.0002¶	37, 38
Fe(TpivPP) (NMeIm)CO	1969	"Irreversible"‖	

* Equilibrium constants involving dissolved gases should be expressed in terms of the partial pressure of the gas *over* the solution, not the concentration of the gas in solution. Because the activity of the dissolved gas is equal to the partial pressure of the gas above the solution, direct comparisons can be made of K_{eq} in various solvents, without regard to gas solubility, *only if* K_{eq} is expressed in terms of pressure.

† These values are typical for a wide range of Mbs and Hbs.

‡ These are the $P_{1/2}^{CO}$ for the binding of the fourth CO and for the overall equilibrium, respectively.

§ P = diethylprotoporphyrinato.

¶ P = deuteroporphyrinato in benzene.

‖ Evacuation at 10^{-5} torr for 75 hr does not affect the intensity of the ν_{CO} bond. Hb Zurich refers to hemoglobin Zurich.

Nature of CO Binding. The infrared stretching frequencies of bound CO in hemes and hemoproteins appear to correlate with the equilibrium constants for binding of CO. More importantly, the hemoprotein structure functions to *reduce* significantly the affinity for CO. Table 2 presents the binding constants and stretching frequencies for a series of Fe porphyrin CO adducts. It has recently been suggested (42) that the ν_{CO} of ferrous porphyrin proteins correlates with the redox potential for the Fe^{III}/Fe^{II} couple. However, this correlation fails to hold in simple porphyrin systems: as noted above, ν_{CO} is not affected by the nature of the porphyrin, whereas $E_{1/2}Fe^{III}/Fe^{II}$ changes by more than 100 mV (43) where $E_{1/2}$ refers to the electrochemical half-wave potential. We suggest that the lowering of ν_{CO} in Mb and Hb from that in simple porphyrins is, at least in part, due to distortion of the linear Fe-CO group from a normal to the porphyrin plane by bulky amino acid residues near the binding site. This tilted conformation is now documented by structure determinations on three diverse hemoproteins: insect (distal residue: Ile) (44), bloodworm (distal residues: Leu, Val)

(45), and sperm whale Mb (distal residues: His, Val) (46). In simple porphyrins the CO should be linear, as in the structure of Fe(TpivPP)(NMeIm)CO (47).

The hypothesis that the ν_{CO} is altered by distortion is supported by several pieces of evidence which indicate that ν_{CO} can be altered by affecting the structure of the binding site of hemoproteins. Denatured Hb in a KBr pellet has ν_{CO} = 1970 cm^{-1} (37), the same as unhindered porphyrins. Mutant Hbs have ν_{CO} approaching those of free porphyrins if the distal histidine is replaced (48): HbZ (β_{63} His → Arg) shows ν_{CO} at 1951 cm^{-1} (the normal α chains) and 1958 cm^{-1} (the β chains) and HbM_{Emory} (β_{63} His → Tyr) at 1951 cm^{-1} and 1970 cm^{-1}. In these mutants the binding site is thought to be more open (49).

Furthermore, the CO stretching frequency and the CO affinity constant appear to correlate. This correlation has been overlooked in the past (48) because the overall affinity of Mb for CO is higher than that of Hb, while the ν_{CO} is reversed: however, because ν_{CO} is probing the *relaxed* conformation of Hb, it is *not* the *overall* CO affinity which one should examine, but that for binding to the relaxed form. This can be approximated by the intrinsic binding constant (including the statistical factor of 4) for binding the fourth CO, $Hb(CO)_3 \rightleftharpoons Hb(CO)_4$, which is 10-fold greater in its affinity for CO than the overall Hb equilibrium constant and hence four times *greater* than that of Mb. In addition, the excellent work of Caughey and associates (36) indicates that hemoglobin Zurich binds CO at least three times stronger in its mutant β chains (ν_{CO} = 1958 cm^{-1}) than in its normal α chains (1951 cm^{-1}). Table 2 gives persuasive data that the lower the ν_{CO}, the lower the affinity for CO. There are, of course, other significant contributions to ν_{CO} (especially the local polarity). The portion which each of these play must, at the moment, remain in doubt.

It is our hypothesis that a major function of the protein is to provide steric bulk to the ligand binding site so as to distort normally linear ligand systems (e.g., Fe-CO). It must be stressed how remarkable it is that three separate hemoproteins with quite different amino acid sequences—even in the immediate vicinity of the binding site—all show distorted CO binding. The effect of this distortion is readily apparent in the CO affinity constants (compare $P_{1/2}^{CO}$ in Table 2). Between the free porphyrin in benzene and Mb there is a 100-fold difference in the binding of CO‡ while there is only a 2-fold difference in the binding of the intrinsically bent O_2^- (14). Other effects of the distal residues (especially histidine) such as increased polarity, hydrogen bonding, or other bonding interactions (53) serve only to stabilize the binding of CO.

The teleological rationale for the reduced CO affinity in hemoproteins stems from the high concentration of CO which biological systems must face. Although the partial pressure of CO, P_{CO}, present in the uncontaminated atmosphere [less than 0.2 ppm (54)] is insignificant even to unconstrained ferrous heme models, the primary source of CO in biological systems in *endogenous:* the use of porphyrins and the production of CO are inherently linked. The biological catabolism of Hb and Mb has been known for more than 20 years to produce 1 mol of CO per heme (55–57) with the production of biliverdin-IXα which undergoes further decomposition to bilirubin, the yellow bile pigment. It is important that this catabolic pathway appears to be a kinetically and thermodynamically intrinsic property of iron porphyrins; even hemin, merely in the presence of ascorbic acid and oxygen, will produce a stoichiometric quantity of CO (57, 58). The catabolism of Hb, Mb, *P*-450, cytochromes, and other porphyrin containing proteins causes, even in air with P_{CO} = 0, roughly 1% of the body's Hb and Mb to be bound as HbCO and MbCO (59). In a closed system, the equilibrium P_{CO} in mammalian tissue is 10–50 ppm (i.e., at this ambient P_{CO} animals show no net loss or gain in [HbCO]), corresponding to 4–5% COHb (56). The tilting of CO in biological oxygen carriers such as Hb and Mb assumes critical importance in light of the inherent CO production from porphyrin catabolism. If Hb and Mb did not have hindered binding sites for CO and if CO were not forced to distort, then the amount of Hb and Mb poisoned by endogenous CO would be considerably greater. For unhindered Fe porphyrins, $[FeCO]/[FeO_2](P_{O_2}/P_{CO})$, commonly referred to as M, is greater than 1500 (from Table 2). With a local P_{CO} about 30 ppm, [HbCO] or [MbCO] would be 20% in the absence of steric hindrance, at which level heavy work becomes difficult and functioning is impaired. This lowered CO affinity of hemoproteins is, of course, independent of any possible correlation with ν_{CO}.

Several experiments are suggested by these observations. Affinity constants for CO for the mutant hemoglobins Hb Zurich and HbM_{Emory} (=$HbM_{Saskatoon}$) or HbM_{Boston} (α_{58} His → Tyr), which apparently have not been measured, would clarify the potential correlation of ν_{CO} with the affinity constant [although the on-rates of recombination have been measured (60, 61) and are much faster than HbA, as expected]. The difficult experiment of measuring ν_{O_2} in such mutant Hbs whose distal pocket is more open than in HbA would shed light on the source of the 50 cm^{-1} difference between our model dioxygen adducts' ν_{O_2} and that of Hb or Mb. If steric constraints are of major importance, then ν_{O_2} for HbZ and the others should approach that of the "picket fence" model; if polarity differences are responsible, then ν_{O_2} of other Fe porphyrin complexes with altered pocket polarities would clarify the situation. The effect of different axial ligands on ν_{O_2} would be of considerable interest as well.

Further work needs to be done on the effect of steric constraint on the axial base to delineate the difference in CO and O_2 binding properties of free porphyrin and base appended porphyrins. One such study is the CO, or preferably O_2, binding to base appended porphyrins with different length attachments. Another approach is to use 2-MeIm or other hindered free bases, which cannot approach the porphyrin plane closely, in ferrous porphyrin ligand affinity studies. In fact, the excellent work of Rougee and Brault (38), in which Fe(deut)(2-MeIm) was found to bind CO some 200 times more poorly than Fe(deut)(Im), is the first piece of evidence that the Hoard-Perutz mechanism of cooperativity (62) is actually capable of explaining the decreased O_2 affinity of Hb in the tensed form.

CONCLUSIONS

The nature of O_2 binding in Hb and Mb and in simple Fe porphyrin models is becoming clear. When O_2 is bound as a bent, rather than a triangular, ligand, it is best described as bound superoxide. This is true for Fe, Co, and perhaps as well for Cr. This bent geometry may be critical to biological functioning because it allows the discrimination between O_2 and CO. The use of steric bulk near the Fe binding site in hemoproteins to distort the FeCO geometry and hence lower its stability (without significantly affecting O_2 bonding) seems essential to avoid massive poisoning from the CO intrinsically formed from porphyrin catabolism.

‡ The only other reported $P_{1/2}^{CO}$ for free porphyrins are of appended base ferrous porphyrins (50, 51). The reported value of 0.2 torr has been revised (T. G. Traylor, personal communication) and is now in agreement with the results of ref. 38. This value is similar to that of microperoxidase (52) ($P_{1/2}^{CO}$ = 0.0004 torr at pH 7, 20°) a biological histidine-tailed ferrous porphyrin.

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