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MODELS FOR COOPERATIVE OXYGEN BINDING IN HEMOGLOBIN

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Abstract - Equilibrium measurements of oxygen binding by iron(II) and cobalt(II) picket fence porphyrins exhibit $p_{\frac{1}{2}}^{O_2}$, $\Delta H°$, and $\Delta S°$ values close to those of myoglobin and cobalt myoglobin respectively. In contrast the CO affinities of simple iron(II) porphyrins are much greater than those of the hemoproteins, hemoglobin (Hb) and myoglobin (Mb). This difference is apparently caused by distal residues in Hb and Mb. With sterically constrained axial bases iron(II) and cobalt(II) picket fence porphyrins exhibit lower oxygen affinities in solution--thus modeling the "T" form of Hb. In the solid state two picket fence iron(II) porphyrins exhibit reversible cooperative oxygen binding.

Recently we have isolated and fully characterized dioxygen adducts of "picket fence" iron porphyrins. Without the agency of a protein these dioxygen complexes are kinetically stable for prolonged periods as crystalline solids and for shorter periods in solution at ambient temperatures. The preparation and characterization of these analogues of the oxygen-binding hemoproteins have been recently reviewed (1). Physical properties of these model dioxygen adducts such as their Mössbauer spectra, magnetic circular dichroism spectra, νO_2 infrared bands, magnetic properties, and structural features about iron are closely congruent with those same features of oxyhemoglobin (HbO_2), and oxymyoglobin (MbO_2).

This account summarizes oxygen binding equilibrium studies of iron and cobalt "picket fence" porphyrins and reveals the apparent relationships between the oxygen affinities of synthetic models and those of the hemoproteins. These results support a putative mechanism by which the protein globin could control oxygen affinity in the limiting "T" and "R" quaternary states of hemoglobin and thus effect cooperativity.

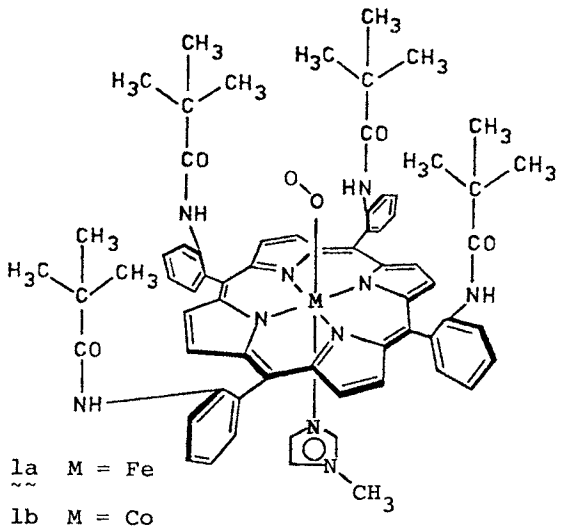
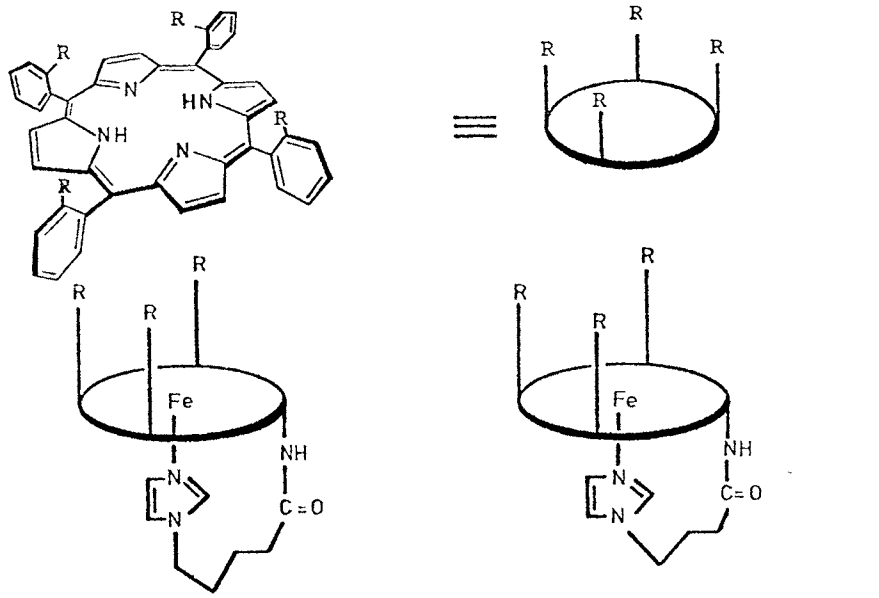


Fig. 1 Picket fence porphyrin dioxygen adducts

Two types of "picket fence" porphyrins (1) have been employed in the present work. First we have used the original "picket fence" porphyrin 1 (Fig. 1) which has a supporting axial base such as N-methyl imidazole (N-MeIm) not directly attached to the porphyrin but coordinated to iron on the side opposite to the protective enclave. The second type (2) has three "pickets" on one side of the porphyrin providing a protective oxygen binding pocket and an axial imidazole bound to the opposite side of the porphyrin, 2. Two variations of the three picket complex, having the axial imidazole joined through five and four carbon chains (2a and 2b), are shown in Fig. 2.



FePF5T

2a Fe(3-picket-5-C-Im)

FePF4T

2b Fe(3-picket-4-C-Im)

R = $NHC(=O)C(CH_3)_3$

Fig. 2 Three picket fence porphyrins

The equilibria involving coordination of axial bases and dioxygen to synthetic porphyrins are illustrated in Fig. 3. Coordination of one axial base (eg. N-MeIm) to the intermediate spin (S=1), four-coordinate iron(II) porphyrin 3 affords a high spin (S=2), five-coordinate complex 4 and is governed by the equilibrium constant K_1. Conversion of the high spin complex 4 into the low-spin (S=0) six-coordinate compound 5 is measured by K_2. Reversible coordination of the high-spin ferrous porphyrin 4 with dioxygen affording the diamagnetic oxygen adduct 6 is the reaction of greatest interest. The oxygen affinity of synthetic porphyrins is measured by the magnitude of the equilibrium constant for this reaction, K_3. Probably because of the spin change, for iron(II) porphyrins, $K_2 > K_1$ and since these are associative equilibria this difference is magnified by lowering the temperature. Thus in the case of iron porphyrins such as 1a, the oxygen binding 5-coordinate state 4 is inevitably a minor component in solution. The equilibrium described by K_2 interferes with direct measurement of the oxygen affinity, K_3. In principle this problem can be overcome by using an appended axial base such as that in complexes such as 2--a technique which was employed earlier by Chang and Traylor (3). However at lower temperatures such tail-base porphyrins exhibit a strong tendency to associate by forming mixed four and six coordinate dimers (4). In the case of cobalt(II) porphyrins the high to low-spin change occurs in K_1 with the result that $K_1 >> K_2$ so that K_3 can be measured directly in solution without interference from K_2, even in the presence of excess axial base.

With the original picket fence porphyrins the complications encountered from interfering equilibria in solution were obviated when we discovered that the

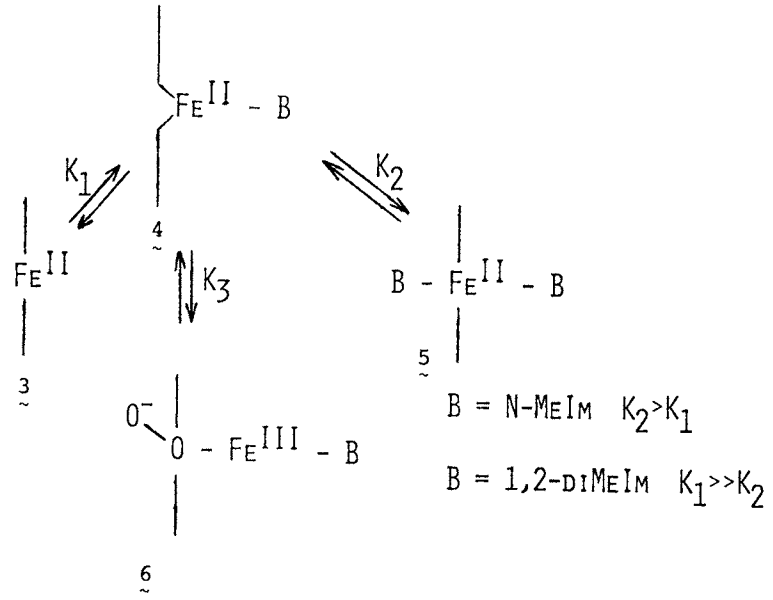


Fig. 3 Equilibria involving axial bases and iron(II) porphyrins

solid iron-oxygen adduct 1a is porous and can be equilibrated with ambient gases. Thus under vacuum all of the iron sites in the solid complex 1a lose oxygen. When the resulting solid high-spin, five-coordinate iron complex is exposed to oxygen, equilibrium is established. Using an electronic manometer and a thermostated apparatus of known volume, the fraction of oxygenated sites, y, can be measured at different pressures of oxygen, p_{O_2}. The solid-gas equilibrium data conform to Langmuir's isotherm (Fig. 4) which is the simple expression describing a system of independent binding sites, behaving in a non-cooperative manner. By determing $p^{O_2}_{\frac{1}{2}}$ (the pressure of oxygen at half saturation; this is the reciprocal of the equilibrium constant K_3) at various temperatures we were able to calculate the entropy changes associated with oxygen binding (5). The analogous solid cobalt(II) picket fence porphyrin behaves similarly, but since the competing equilibrium K_2 is not significant in the case of cobalt we were also able to obtain good solution equilibrium data by determining electronic spectral changes at various partial pressures of oxygen (6). The "tail-based" iron picket fence porphyrins 2a and 2b also bind oxygen reversibly in solution at 25°C. Since in these compounds there are no competing equilibria at temperatures >0°C, it has been possible to measure oxygen affinities for 2a and 2b in solution over a range of temperatures.

$$Fe + O_2 \underset{}{\overset{K_3}{\rightleftharpoons}} FeO_2 \qquad K_3 = \frac{[FeO_2]}{[Fe]p_{O_2}}$$

$$\text{AT } p^{O_2}_{\frac{1}{2}} \quad [Fe] = [FeO_2] \qquad K_3 = \frac{1}{p^{O_2}_{\frac{1}{2}}}$$

$$Y = \frac{[FeO_2]}{[Fe]+[FeO_2]} \qquad p_{O_2} = p_{\frac{1}{2}}\left(\frac{Y}{1-Y}\right)$$

(LANGMUIR ISOTHERM)

Fig. 4 Oxygen binding equilibria

Equilibrium data for the original "picket fence" porphyrin iron complex 1, its cobalt analogue, 1b, and the "tail-based" iron picket fence complex, 2a, are listed in Table 1, along with comparative literature values for the "unconstrained" hemoproteins, myoglobin (Mb), and "R" hemoglobin (Hb(R)) as well as the corresponding cobalt substituted hemoproteins. Table 1 also lists oxygen binding parameters for a simple, unprotected cobalt(II) porphyrin.

TABLE 1. Oxygen affinities for synthetic porphyrins and unconstrained hemoproteins

System	$p_{\frac{1}{2}}^{O_2}(^{25°}_{torr})$	$\Delta H°(^{Kcal}_{m-1})$	$\Delta S°$(eu)
Hb(R)[a] human (4th)	0.96	--	--
Mb[b] (ox)	0.9	-15	-37
Fe(picket fence)(N-MeIm)[c]	0.5	-15.6	-38
Fe(3-picket,5-C-Im)[c]	0.6	-16.3	-40
Fe(3-picket,4-C-Im)[c]	0.6	-16.7	-42
CoHb(R) (4th)[d,e]	45	--	--
CoMb sperm whale[e]	57	-13.3	-40
$CoPP_{IX}$(N-MeIm)[f]	1.5×10^4	-10.6	-41
Co(picket fence)(N-MeIm)[c]	61	-13.3	-40
Co(picket fence)(N-MeIm)[c]	140	-12.2	-38

(a) G. Ilgenfritz and T. M. Schuster, J. Biol. Chem., 249, 2959 (1970).
(b) Reference 13.
(c) This work: K. Doxsee, T. Halbert, and K. Suslick.
(d) Extrapolated from data at 15°.
(e) Reference 14.
(f) H. C. Stynes and J. A. Ibers, J. Amer. Chem. Soc., 94, 1559 (1972).

The data in Table 1 show that the oxygen affinities ($p_{\frac{1}{2}}^{O_2}$) and the thermodynamic parameters measured for our iron(II) picket fence complex 1a are remarkably similar to those reported for the unrestrained, naturally occuring hemoproteins. Similarly the cobalt picket fence porphyrin, 1b, either in the solid state or in solution shows oxygen affinities very much like those of the cobalt substituted hemoproteins. In each case the oxygen affinity of the iron derivative is about 100 times greater than that of the cobalt analogue. The solution and the solid state oxygen affinities for the iron picket fence complexes 2a and 1a are virtually the same and for the cobalt picket fence complex 1b these show only a two-fold difference. On the other hand the simple, unprotected cobalt(II) porphyrins exhibit a much lower affinity for oxygen than the cobalt substituted picket fence porphyrin 1b or the hemoprotein (CoMb) (less by a factor of 300). This difference appears to result from solvation effects (7). Apparently the picket fence enclosure provides a fixed microsolvent environment. Whether or not the polarity of the picket fence amide groups in the oxygen binding pocket substantially influences the oxygen affinity remains an unanswered question.

A major conclusion to be drawn from the data in Table 1 is that the model iron compounds exhibit quantitatively the same affinities for oxygen as the natural, unrestrained hemoproteins. This may simply reflect the intrinsic oxygen affinity of a ferrous porphyrin having an axial imidazole ligand in the absence of solvation effects. In this regard it is interesting that the high-affinity "R" state of hemoglobin and all mammalian myoglobins exhibit virtually the same oxygen affinities.

It is important to note that our model compounds do not have substituents analogous to the hemoprotein "distal" groups which are known to be positioned directly over the oxygen binding site in all normal hemoglobins and myoglobins. The function, if any, of the distal histidine E-7 has long been controversial (8). However since mutants which lack histidine E-7 have oxygen affinities very similar to those of normal hemoproteins, this distal group cannot bond to and thus stabilize coordinated dioxygen. A recent neutron diffraction study of sperm whale MbCO (9) shows that the imidazole N-H group in His-E7 does not hydrogen bond to the CO group and presumably would not do so with dioxygen.

A clue to the probable function of these distal residues became evident when it was noted (10) that the synthetic model compounds bind carbon monoxide about 100 times more strongly than the hemoproteins. Representative equilibrium data are presented in Table 2. X-ray diffraction studies of these model CO adducts show the expected linear Fe-C-O group oriented normal to the porphyrin plane (1). However several recent X-ray diffraction studies of hemoprotein CO adducts show that the Fe-C-O group is either non linear (bent) or is linear but tilted off the normal to the porphyrin plane (9, 11). A similar effect has been found in a met cyano hemoprotein (11c). Two distal amino acids, valine E-11 and histidine E-7, are positioned so that these distal residues distort coordinated carbon monoxide from its normal geometry. On the basis of structural studies on our model complex the dioxygen group in HbO_2 would be expected to be bound to iron in an angular manner and thus not to experience any non-bonded interaction with these distal residues. Thus we have proposed that the role of these distal residues is to discriminate between the equilibrium binding of dioxygen and carbon monoxide by sterically distorting the latter (10). Such discrimination is required since the catabolism of heme produces an equivalent of carbon monoxide which is thus an endogenous poison that all aerobic organisms must endure. Without such distal groups hemoglobin and myoglobins would be binding CO to an extent (~25%) that would impair their function.

TABLE 2. Structural effects on CO binding

Substance	$p_{\frac{1}{2}}^{CO}(25°_{torr})$	∢Fe-C-O
Fe(deuteroporphyrin)(Im)[a]	2.4×10^{-4}	(180°?)
Microperoxidase[b]	4×10^{-4}	(180°?)
Fe(picket fence)(N-MeIm)[c]	"very small"	180°
Mb[d]	1.8×10^{-2} (horse)	135° (whale)
Hb[d]	3.5×10^{-2}(av) (human)	140° (horse)
Chironomus thummi[e]	4×10^{-3}	145±15°

(a) M. Rougee and D. Brault, Biochemistry, 14, 4100 (1975) and ref. therein.
(b) V. S. Sharma, H. M. Ranney, H. M. Geibel, J. F. Traylor, and T. G. Traylor, Biochem. Biophys. Res. Commun., 66, 1301 (1975).
(c) Reference 10.
(d) Reference 13.
(e) G. Amiconi, E. Antonini, M. Brunori, H. Formaneck, and R. Huber, Eur. J. Biochem., 31, 52-58 (1972).

Perhaps the most remarkable, physiologically essential, and yet controversial property of hemoglobin is cooperativity (8). As tetrameric Hb begins to bind oxygen the affinity of the individual heme units for oxygen rises. Thus there is a cooperative interaction between the relatively distant heme groups transmitted through the protein. Both the phenomenon of cooperativity and one of its major physiological uses are illustrated by the oxygen binding curves shown in Fig. 5. The curve for myoglobin is the hyperbole expected from the

simple equilibrium binding to independent heme groups--as expressed by Langmuir's isotherm. On the other hand, hemoglobin shows a sigmoid curve characteristic of cooperative ligand binding. At the lower oxygen pressures which are characteristic of muscle tissue, hemoglobin shows a much lower affinity than myoglobin and thus under these conditions hemoglobin releases oxygen to myoglobin. At the higher oxygen tension typically found in the lungs and in arteries, hemoglobin exhibits an oxygen affinity close to that of myoglobin. These features are essential to the transfer and transport of oxygen.

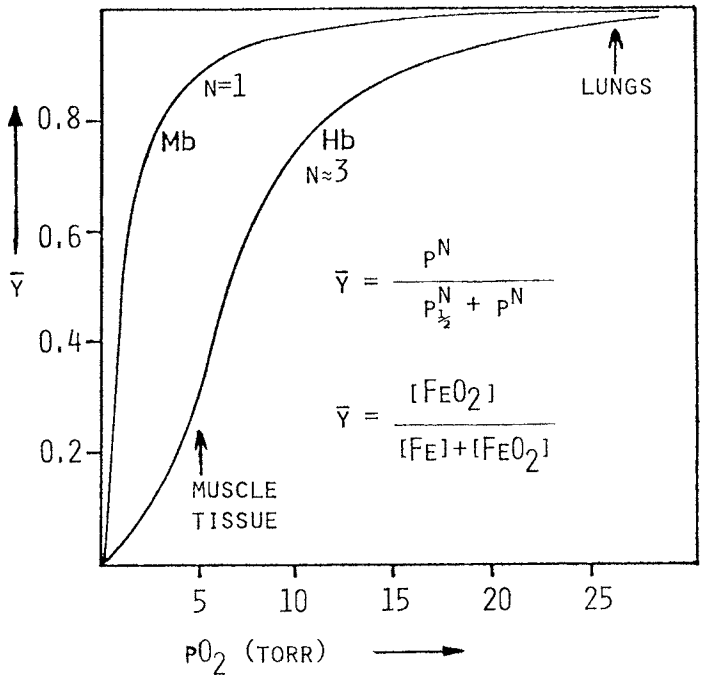


Fig. 5 Hb and Mb oxygen binding curves

The structural origin of cooperativity has been elucidated by Perutz (8) who showed that this change in oxygen affinity is associated with a change in quaternary protein structure. The major features of Perutz's proposal are outlined in Fig. 6. The unliganded quaternary state of hemoglobin, the "T" form, exhibits a diminished affinity for oxygen because the protein restrains the imidazole ligand of the "proximal" histidine F_8 from moving towards the porphyrin plane as iron binds dioxygen on the opposite, "distal", side of the porphyrin. On the basis of stereochemical arguments Hoard (12) had earlier predicted that the high-spin unligated ferrous iron would be displaced well away from the plane of the porphyrin but would move into the porphyrin plane upon oxygenation. Hoard's predictions have been largely confirmed by structural studies on the hemoproteins and model porphyrin complexes. It is estimated that the imidazole nitrogen which is coordinated to iron moves about 0.6Å closer to the mean plane of the porphyrin upon oxygenation (8). Restraining the axial imidazole ligand is equivalent to lowering its ligand field strength in the oxygenated form. In the "T" state of Hb the protein conformation apparently locks the imidazole of the proximal histidine F_8 into the equilibrium deoxy position. In the "R" quaternary state the protein is relaxed and the imidazole is free to assume its equilibrium geometry from iron and the porphyrin plane in either the oxygenated or the unoxygenated state. Thus the oxygen affinity of the individual hemes in the "R" state of hemoglobin should be approximately that of myoglobin. The "T" quaternary state is stabilized by organic phosphate esters and by protons, thus accounting for the effect of those reagents on cooperativity. The "R" state seems to be stabilized by ligands such as oxygen, carbon monoxide, and nitric oxide all of which tend to pull iron into the porphyrin plane and to exhibit cooperative binding to hemoglobin (8).

The cooperative behavior of hemoglobin is better analyzed by plotting the oxygen binding curve with the logarithmic Hill equation (13) as illustrated in Fig. 7. Three linear regions are found in Hb Hill plots--the

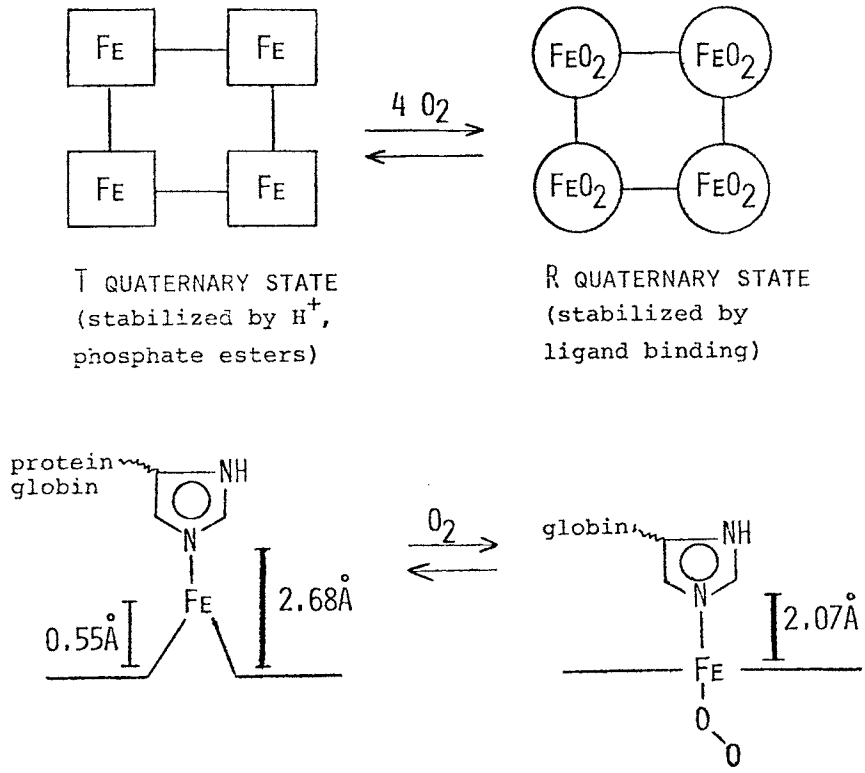


Fig. 6 Hemoglobin cooperativity (Perutz, Hoard)

first and third having a slope of unity and the second a slope of about 3. The first region describes the oxygen binding by the low affinity "T" form and the third by the high affinity, myoglobin-like, "R" form. The second linear region describes oxygen binding in the ill-defined intermediate range. The slope of this second linear region, n, is termed the Hill coefficient and its magnitude is often cited as a measure of cooperativity. However a more realistic measure of cooperativity is the ratio of the intrinsic affinities of the individual hemes in the "T" and "R" states--corrected for the statistical factors. The ratio of oxygen affinities of the individual hemes in the "R" and "T" states is roughly 100:1 for Hb and 8:1 for cobalt hemoglobin (14). The movement of the proximal histidyl N in CoHb is ~0.4Å compared with ~0.6Å in Hb (7).

We have devised simple synthetic models for the "T" state of Hb and CoHb (6). These models make use of 2-methyl-imidazole and 1,2-dimethyl-imidazole and take advantage of the steric interaction between the 2-methyl substituent and the porphyrin plane. Such steric repulsion tends to hold these axial bases away from the porphyrin plane and to restrain them in this position as the metal is oxygenated. These models for the "T" state are schematically represented in Fig. 8. Earlier we used this technique to crystallize and structurally characterize, 2-methylimidazole-tetraphenyl-porphyrinato-iron(II), the first synthetic analogue of deoxymyoglobin (15).

Oxygen affinities of our "T" and "R" models are compared with those of "T" and "R" Hb and CoHb in Table 3 (6,16). Note that good solution oxygen binding data can be obtained for iron picket fence porphyrins using 1,2-dimethylimidazole since a second equivalent of this hindered axial base cannot strongly bind to iron ($K_1 >> K_2$ in Fig. 3). The ratio of oxygen affinities for our T and R model iron complexes is ~80:1 compared with ~100:1 for Hb. The corresponding values for our cobalt analogues are 6:1 compared with 8:1 for CoHb (6). This close correspondence in O_2 affinities is happenstance: more sterically hindered imidazoles (e.g., 1,2,4,5-tetramethyl-imidazole) should provide even less O_2 affinity. Thermodynamic parameters have been

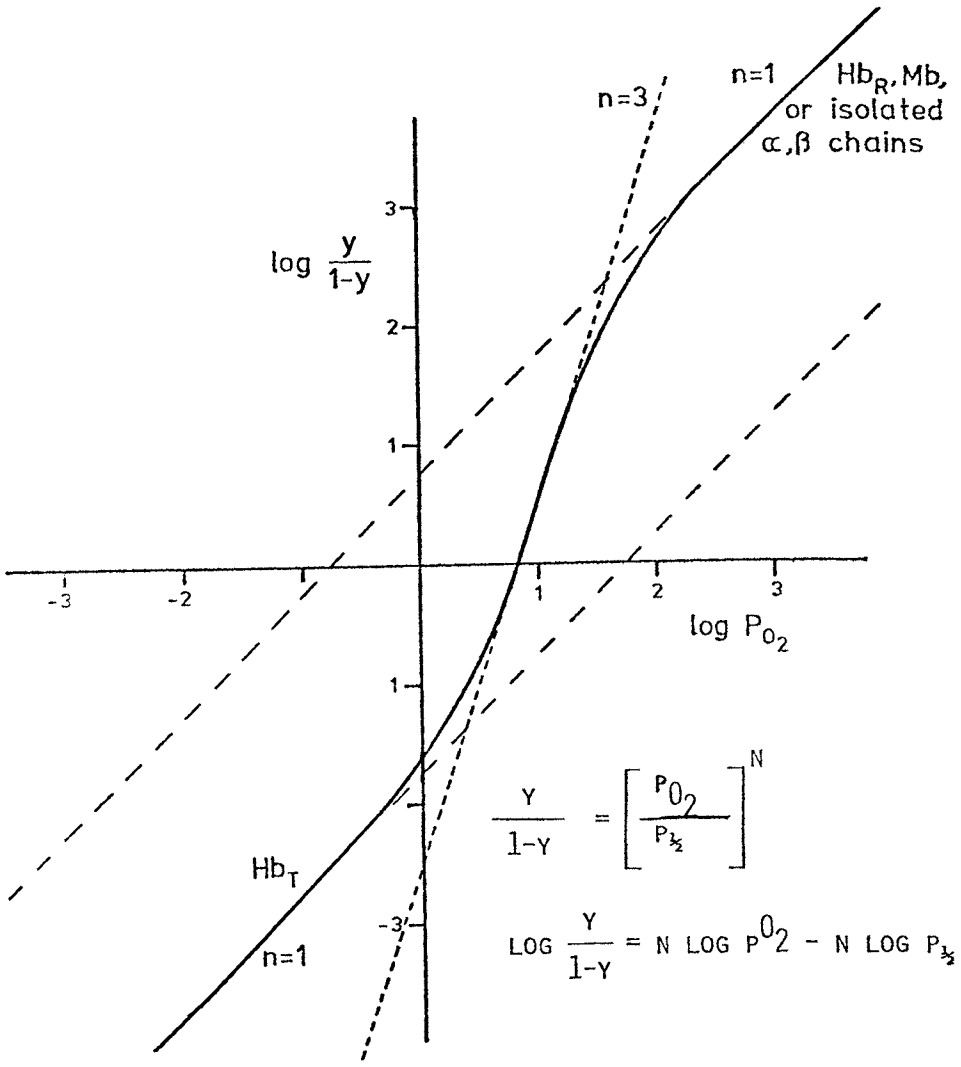
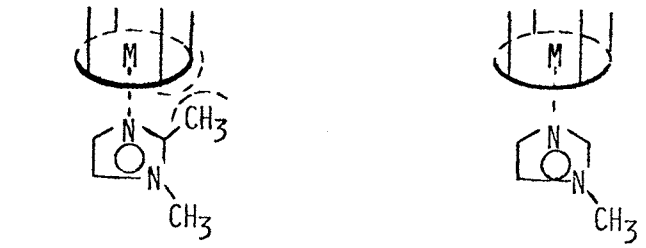


Fig. 7 Representative Hill plot showing cooperative Hb O_2 binding



MODEL FOR "T" STATE
M(PICKET FENCE)(1,2-DIMEIM)

MODEL FOR "R" STATE
M(PICKET FENCE)(N-MEIM)

Fig. 8 Picket fence porphyrin models for Hb T and R states

measured for our T and R model compounds. These indicate that for the iron derivatives differences in affinities arise from the enthalpy rather than the entropy term. For the cobalt derivatives these changes are too small to make a clear distinction. Nevertheless, in the absence of solvation effects, the affinity difference is expected to arise from the enthalpy term.

TABLE 3. Models for R and T states of Hb

Substance	$p^{O}_{\frac{1}{2}}2$ (25°, torr)
Hb (R)	0.15 to 1.5[a]
Hb (T)	9 to 160[a]
Fe(picket fence)(N-MeIm)	0.5[b] (solid state)
Fe(3-picket-5C-Im)	0.6[b] (toluene)
Fe(picket fence)(1,2-diMeIm)	38[b] (toluene)
CoHb (R)	20 to 125[a,c]
CoHb (T)	160 to 800[a,c]
Co(picket fence)(N-MeIm)	70 (toluene)[b,c]
Co(Picket fence)(1,2-diMeIm)	450 (toluene)[b,c]

(a) Reference 14.
(b) This work: K. Doxsee, T. Halbert, E. Rose, and K. Suslick.
(c) 15°.

Having previously used solid-gas equilibrium data to great advantage in our earlier study of picket fence iron porphyrins (5), we then employed this technique to examine the picket fence iron(II) complex with the sterically restrained axial base, 2-methyl-imidazole (16). The results from this experiment are amazing. It soon became apparent that the data do not conform to a simple Langmuir isotherm. However when these data are plotted with the Hill equation, the result shows clear cut evidence for reversible, cooperative oxygen binding. Solid-gas Hill plots for the two hindered axial bases, 1,2-dimethyl-imidazole and 2-methyl-imidazole, are illustrated in Fig. 9, along with the simple 1-methyl-imidazole non-cooperative case. These solid-gas cooperative binding curves have been shown to be completely reversible and have been reproduced with independently prepared samples. The measured $p^{O}_{\frac{1}{2}}2$ values and Hill coefficients are listed in Table 4. For the 2-methyl-imidazole case the low and high affinity forms differ by a factor of only 20 with a Hill coefficient of 2.6. Preliminary structural studies of this deoxy complex by Ibers and Molinaro (17) show four molecules in the unit cell but so far give no clue as to the structural origin of this reversible solid-state cooperative oxygen binding. It is hoped that further structural work on the liganded state will reveal the detailed molecular origin of this cooperativity. It is clear that oxygenation of some of the iron sites must induce a reversible change in the crystal lattice.

Comparison of the properties of model oxygen complexes with those of their hemoprotein analogues reveals several roles which the protein in Hb influences the chemistry of heme. Thus the protein: (a) kinetically stabilizes the dioxygen complex, (b) enforces 5-coordination in the deoxy state, (c) prevents endogenous carbon monoxide poisoning by lowering the intrinsic CO affinity through steric interactions with the "distal" residues, and (d) lowers O_2 affinity in the "T" quaternary state of Hb--apparently by restraining the proximal histidine. These model studies may in the near future clarify in part the mechanism by which the change from "T" to "R" quaternary states is carried out. Finally, it seems clear that any synthetic oxygen carrier which is to be used as a blood substitute must emulate most if not all of these properties which are characteristic of the Hb, as well as avoiding obvious and serious toxicity problems.

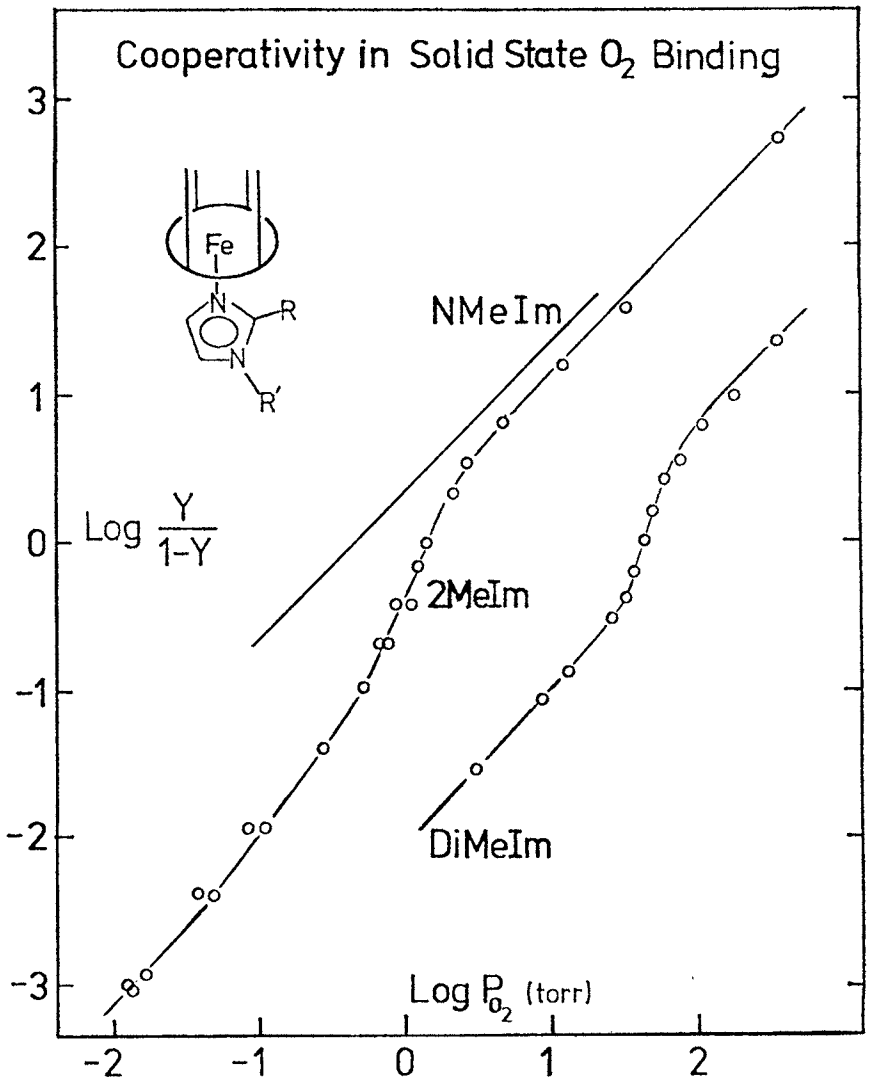
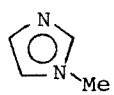


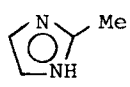
Fig. 9 Solid-gas equilibrium curves

TABLE 4. Cooperativity in O_2 binding in the solid state, 25°[a]

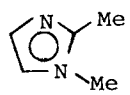
System	High Affinity p^{O_2} (torr)	Low Affinity p^{O_2} (torr)	Hill Coefficient, n
Fe(picket fence)(N-MeIm)	0.5 (non-coop.)		1.0
Fe(picket fence)(2-MeIm)	0.7	14	2.6
Fe(picket fence)(DiMeIm)	14	112	3.0



NMeIm



2MeIm



DiMeIm

(a) This work, K. Suslick.

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