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Chemistry

Model compounds for the T state of hemoglobin

(cobalt-substituted hemoproteins/cooperativity/hemoproteins/myoglobin/oxygen binding)

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ABSTRACT **O_2 binding to a series of ferrous and cobaltous "picket fence" porphyrins is reported. *N*-Methylimidazole and covalently attached imidazoles give O_2 binding to ferrous porphyrins with $\Delta H° = -16.2$ kcal/mol (−67.7 kJ/mol) and $\Delta S° = -40$ eu (standard state, 1 atmosphere O_2). Similar studies with cobaltous porphyrins yield $\Delta H° = -12.8$ kcal/mol (−53.5 kJ/mol) and $\Delta S° = -39$ eu. These values match well those of myoglobin and isolated subunits of hemoglobin and their cobalt reconstituted analogues. 1,2-Dimethylimidazole has been successfully used to mimic the presumed restraint of T state hemoglobin. In direct analogy to the decreased cooperativity shown by cobalt-substituted hemoglobin, model cobalt porphyrins show a smaller decrease in O_2 affinity than the analogous iron porphyrins when the axial base is hindered. Thermodynamic data are presented. The molecular mechanism of cooperativity in hemoglobin is discussed.**

The mechanism of cooperativity in hemoglobin (Hb) is of continuing interest (1–3). Model porphyrins capable of reversible oxygen binding have contributed to a fuller understanding of myoglobin (Mb) and other monomeric hemoproteins (4–6). Very little work, however, has appeared on models for the low affinity, T state, of Hb. The effect of steric restraint built into the porphyrin (7, 8) or the axial base (9–13) has not been fully explored. We wish to report a full study of O_2 binding to both iron and cobalt "picket fence" porphyrins with hindered and unhindered imidazoles. In addition, we make here a preliminary report on the synthesis and characterization of "picket fence" porphyrins with covalently attached axial bases.

MATERIALS AND METHODS

meso-Tetra($\alpha,\alpha,\alpha,\alpha$-*o*-pivalamidophenyl)porphyrin (H_2T-PivPP) was prepared as described (4). Cobalt was inserted by use of anhydrous $CoCl_2$ in a solution of tetrahydrofuran with a trace of 2,6-lutidine at 50° under N_2. Further purification consisted of chromatographic separation on Woelm neutral alumina. Details are presented elsewhere (14). *meso*-Tri(α,α,α-*o*-pivalamidophenyl)-β-*o*-3-(*N*-imidazolyl)propylamidophenylporphyrin [Piv$_3$(4CImP)Por] and *meso*-tri(α,α,α-*o*-pivalamidophenyl)-β-*o*-4-(*N*-imidazolyl)butylamidophenylporphyrin [Piv$_3$(5CImP)Por] were prepared by the reaction of 4-(*N*-imidazolyl)butyl chloride and 5-(*N*-imidazolyl)valeryl chloride, respectively, with *meso*-tri(α,α,α-*o*-pivalamidophenyl)-β-*o*-aminophenylporphyrin. Extreme care must be taken to prevent exposure of the porphyrins to O_2 and light due to singlet molecular oxygen production catalyzed by the metal-free porphyrin (15) and trapping by the attached imidazole (16). Chemical structures of these porphyrins are shown in Fig. 1. The *meso*-tri(α,α,α-*o*-pivalamidophenyl)-β-*o*-aminophenylporphyrin was prepared by the reaction of the readily available *meso*-tetra($\alpha,\alpha,\alpha,\alpha$-*o*-aminophenyl)-porphyrin (4) with limited amounts of pivaloyl chloride [$(CH_3)_3COCl$] and isolated by column chromatography. Iron was easily inserted under inert atmosphere by use of anhydrous $FeBr_2$ in 1:1 benzene/tetrahydrofuran with a trace of 2,6-lutidine, followed by chromatographic filtration through a short plug of alumina. All intermediates and porphyrins described above have been well characterized by elemental analysis, UV/visible, nuclear magnetic resonance, magnetic circular dichroism, and Mössbauer spectroscopies, and purities demonstrated with high-pressure liquid chromatography. Details of these syntheses will be presented elsewhere.

All solvents were distilled and stored under N_2: toluene from Na metal, tetrahydrofuran from CaH_2, *N*-methylimidazole (NMeIm) vacuum-distilled from KOH, 1,2-dimethylimidazole (Me_2Im) vacuum-distilled from Na metal, and 2,6-lutidine passed through alumina and distilled from $BF_3{\cdot}Et_2O$. Anhydrous powdered $CoCl_2$ (Alfa) was heated at 100° under reduced pressure for 30 min before use. Anhydrous $FeBr_2$ was prepared in the usual fashion (17). All experimental operations requiring an inert atmosphere were carried out in a Vacuum Atmospheres "Dri-Lab" under N_2.

Oxygen binding equilibria were determined with an apparatus consisting of a 10-mm cuvette with gas inlet and outlet tubes attached to a pair of calibrated Matheson 600 rotameters which mixed pure N_2 with pure O_2 or with premixed O_2 in N_2 mixtures (Liquid Carbonic certified gas mixtures, 5.01 ± 0.20% and 0.140 ± 0.006% O_2 in N_2). Further details are presented elsewhere (14). Concentrations of metalloporphyrins in all cases were ~50 μM. For CoTPivPP, concentrations of NMeIm and Me_2Im were chosen to provide >90% five-coordinate cobalt porphyrins, based on equilibrium constants for axial base ligation determined under N_2 by standard spectrophotometric techniques (18).

RESULTS

Because oxygen binding at temperatures above 0° is incomplete even at 760 torr (101 MPa) O_2, a mathematical approach that does not require knowledge of the spectrum of the pure oxy-

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Abbreviations: Hb, hemoglobin; Mb, myoglobin; CoHb, apohemoglobin reconstituted with Co protoporphyrinate IX; CoMb, apomyoglobin reconstituted with Co protoporphyrinate IX; Me_2Im, 1,2-dimethylimidazole; NMeIm, 1-methylimidazole; TPivPP, *meso*-tetra($\alpha,\alpha,\alpha,\alpha$-*o*-pivalamidophenyl)porphyrinate; Piv$_3$(4CImP)Por, *meso*-tri(α,α,α-*o*-pivalamidophenyl)-β-*o*-3-(*N*-imidazolyl)propylamidophenylporphyrinate; Piv$_3$(5CImP)Por, *meso*-tri(α,α,α-*o*-pivalamidophenyl)-β-*o*-4-(*N*-imidazolyl)butylamidophenylporphyrinate; $P_{1/2}$, O_2 pressure at half-saturation.

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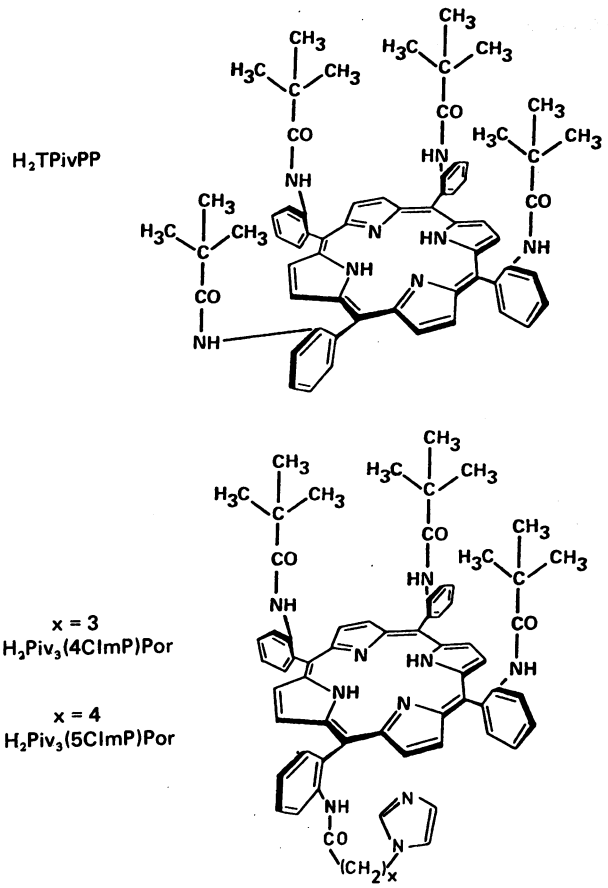


FIG. 1. "Picket fence" porphyrins.

genated complex was used. This approach is a modification of one by Drago (18), who has shown:

$$K^{-1} = P_{O_2}\left[\frac{[MP]_T b\Delta\epsilon}{\Delta A} - 1\right]$$

where K is the equilibrium constant for O_2 binding to the Fe^{II} or Co^{II} porphyrin complex, $[MP]_T$ is the total metalloporphyrin concentration, b is the pathlength of the cell, ΔA is the difference between the absorbance of the solution at oxygen pressure P_{O_2} and the absorbance of the solution in the absence of oxygen, and $\Delta\epsilon$ is the difference between molar extinction coefficients of the oxy and the deoxy complexes at the wavelength chosen. Rearranging the equation gives:

$$P_{O_2} = [MP]_T b\Delta\epsilon(P_{O_2}/\Delta A) - K^{-1}$$

from which it is clear that, since $[MP]_T b\Delta\epsilon$ is a constant, a plot of P_{O_2} against $P_{O_2}/\Delta A$ should be a straight line with slope $[MP]_T b\Delta\epsilon$ and intercept $-K^{-1}$.

Sets of spectra were recorded at each temperature over a wide range of P_{O_2}, and plots of P_{O_2} against $P_{O_2}/\Delta A$ were constructed for two to four wavelengths. Straight lines were then computer fit by a linear least squares program, and equilibrium constants were determined from the intercepts (the standard deviation of these intercept values was generally less than 5%). In all cases, good isosbestic points were observed.

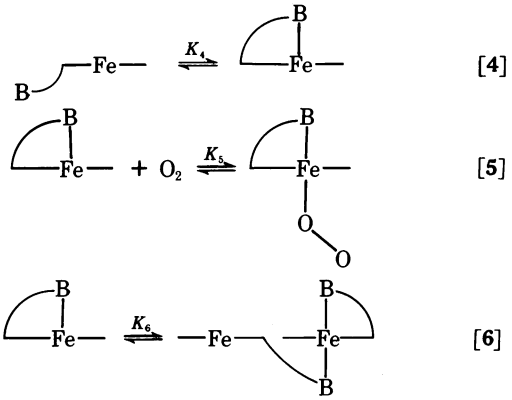
With the simple metalloporphyrins, those without appended axial base, the major solution equilibria that must be considered between the metalloporphyrins (MP) and axial bases (B) and O_2 are:

$$MP + B \overset{K_1}{\rightleftharpoons} MP \cdot B \qquad [1]$$

$$MP \cdot B + O_2 \overset{K_2}{\rightleftharpoons} MP \cdot B \cdot O_2 \qquad [2]$$

$$MP \cdot B + B \overset{K_3}{\rightleftharpoons} MP \cdot B_2 \qquad [3]$$

Since the reaction of interest in this study is the oxygenation reaction [2], solution conditions must be chosen under which the predominant species is MP · B prior to addition of oxygen. With Co^{II}TPivPP in toluene solution under N_2 at 20°C, K_1 is found to be $\sim 1.7 \times 10^4\ M^{-1}$ for NMeIm and $\sim 1.4 \times 10^3\ M^{-1}$ for Me_2Im. With Fe^{II}TPivPP, K_1 is $\sim 3.7 \times 10^4\ M^{-1}$ for Me_2Im under the same conditions. In no case was any evidence found for formation of six-coordinate MP·B_2 (reaction 3), and K_3 must therefore be <10. These observations are in keeping with previous work on the binding of imidazoles to cobalt porphyrins (9) and the binding of hindered imidazoles to iron porphyrins (11–13). In these cases, since $K_1 \gg K_3$, solutions in which the predominant species is the desired MTPivPP(B) can be prepared by judicious use of excess B. For ferrous porphyrins with unhindered imidazoles, $K_3 > K_1$ (10, 11, 13), which precludes the ability to prepare, in solution, FeTPivPP(B). This is the *raison d'etre* of appended axial bases. With the "tailed"porphyrins, those with appended axial bases, the major equilibria are best presented in schematic form:



These are, of course, the close analogues of K_1, K_2, and K_3 of the simple metalloporphyrins. In the "tailed"porphyrins described above, K_4 is very large, i.e., practically no four-coordinate iron is found in solution over the temperature range studied. The dimerization equilibrium of K_6 has been well noted before (8), and is favored by low temperatures and high concentrations. In the case of our "tailed" porphyrins, such dimerization becomes appreciable at <−25° and $>10^{-3}$ M; however, in the temperature and concentration ranges used in these studies, this dimerization is negligible.

By the techniques already described, equilibrium constants K_2 and K_4 were obtained. $\Delta H°$ and $\Delta S°$ were then derived from linear least square fits to standard van't Hoff plots over as wide a temperature range as possible (generally >40°). The data used in these plots follow as pairs of temperature (°C) and $P_{1/2}$ (half-saturation pressure, in torr) from studies in toluene solutions: $FePiv_3$(5ClmP)Por, (35.8°, 1.70), (26.6°, 0.86), (25.2°, 0.60), (19.0°, 0.262), (13.0°, 0.179), (6.3°, 0.094), (1.5°, 0.0516), and (−5.2°, 0.0346); $FePiv_3$(4ClmP)Por, (20.2°, 0.376), (19.1°, 0.328), (13.4°, 0.177), (8.5°, 0.128), (3.3°, 0.0744), (0.2°, 0.048), (−3.0°, 0.0336), (−3.6°, 0.0278), and (−3.9°, 0.0262); FeTPivPP(Me_2Im), (40.3°, 96.8) (34.4°, 85.4), (24.8°, 41.6), (24.1°, 34.6), (5.8°, 6.42), (−7.3°, 1.90), and (−10.1, 1.49); CoTPivPP(NMeIm), (27.0°, 179) (24.9°, 164), (24.7°, 150), (8.2°, 47.2), (−2.8°, 18.7), (−12.6°, 9.53), and (−12.8°, 6.98); and

Table 1. Thermodynamic values for O_2 binding to "R" and "T" state models

System	$P_{1/2}$ (25°), torr*	$\Delta H°$, kcal/mol†	$\Delta S°$, eu†‡
FeTPivPP (NMeIm) solid state§	0.49	−15.6 ± 0.2	−38 ± 1
$FePiv_3$(4CImP)Por toluene solution	0.60	−16.8 ± 0.5	−42 ± 2
$FePiv_3$(5CImP)Por toluene solution	0.58	−16.3 ± 0.8	−40 ± 3
FeTPivPP(Me_2Im) toluene solution	38	−14.3 ± 0.5	−42 ± 2
CoTPivPP(NMeIm) solid state	61	−13.3 ± 0.9	−40 ± 3
toluene solution	140	−12.2 ± 0.3	−38 ± 1
CoTPivPP(Me_2Im) toluene solution	900	−11.8 ± 0.4	−40 ± 2

* Interpolated from $\Delta H°$ and $\Delta S°$; estimated errors ±5%.
† Error limits given are the standard deviations of van't Hoff plots.
‡ Standard state, 1 atmosphere O_2.
§ Ref. 19.

CoTPivPP(Me_2Im), (24.9°, 960), (24.5°, 777), (4.9°, 239), (−12.3°, 52.4), (−13.8, 50.9), and (−15.8, 33.5). These data are summarized, along with comparable data from hemoprotein systems, in Tables 1, 2, and 3. A graphic compilation of these data is presented in Fig. 2.

DISCUSSION

The data presented in Table 1 reveal several interesting points. Clearly, the oxygen binding by the iron porphyrins with unhindered axial bases (e.g., NMeIm or our "tailed" imidazoles) are nearly identical: overall, one may say that $P_{1/2}$ at 25° ≈0.6 torr and that $\Delta H° = -16.2 \pm 0.6$ kcal/mol (−67.7 ± 2.5 kJ/mol) and $\Delta S° = -40 \pm 2$ eu (standard state 1 atmosphere O_2) for all cases. These values compare well with the $\Delta H°$ of O_2 binding by myoglobins and isolated chain Hb, which range from −16.4 to −13.2 kcal/mol and the associated $\Delta S°$, which range between −41 and −32 eu (27) (standard state 1 atmosphere). As noted before with the solid state binding of O_2 by FeTPivPP(NMeIm) (19), the intrinsic O_2 affinity of these simple porphyrins is at least as good as that of the native hemoproteins. These model systems do not have the possibility of hydrogen bonding or electron pair donation to the bound oxygen, nor is the binding pocket particularly polar, nor is it shaped to fit the bound O_2, nor does it have any other particularly unusual characteristics (4). To the extent that these statements are true, we may then view the oxygen affinities of both model systems and relaxed hemoproteins (e.g., Mb, isolated chain Hb, and R state Hb) as originating solely from the ferrous porphyrin imidazole system. In contrast, the carbon monoxide affinities are much lower in most hemoproteins than in the models due to the steric constraints of the hemoprotein binding pocket and the biological necessity to decrease the CO affinity relative to O_2 (28).

Table 2. O_2 affinities of iron porphyrins and hemoproteins

System	Physical state	$P_{1/2}$(25°), torr	Ref.
Mb, sperm whale	pH 8.5	0.70	20
Hb			
α chains	pH 7.5,	0.63	21, 22
β chains	0.1 M phosphate	0.25	
Hb (human, "R")*	Various†	0.15–1.5†	23
$FePiv_3$(4CImP)Por	Toluene solution	0.60	This work
$FePiv_3$(5CImP)Por	Toluene solution	0.58	This work
FeTPivPP(NMeIm)	Solid state	0.49	19
Hb (human, "T")*	Various†	9–160‡	23
FeTPivPP(Me_2Im)	Toluene solution	38	This work

* These are actually the first and fourth intrinsic $P_{1/2}$ values.
† Imai's conditions included various combinations of 0.1 M NaCl, 0.1 M phosphate, 2 mM inositol hexaphosphate, 2 mM 2,3-diphosphoglycerate, all at pH 7.4.
‡ The ratio of these "R" and "T" affinities also varied as a function of conditions, from around 40 to 500.

Table 3. O_2 affinities of cobalt porphyrins and cobalt hemoproteins

System	Physical state	$P_{1/2}$ (15°), torr	Ref.
CoMb (sperm whale)	0.1 M phosphate, pH 7	30	24, 25
CoHb (isolated chains)	0.1 M phosphate, pH 7.4	25	26
CoHb (human, "R")*	Various†	16–180‡	23
CoTPivPP(NMeIm)	Solid state	28	This work
	Toluene solution	70	This work
CoHb (human, "T")*	Various†	120–500‡	23
CoTPivPP(Me_2Im)	Toluene solution	450	This work

* These are actually the first and fourth intrinsic $P_{1/2}$ values.
† Imai's conditions included various combinations of 0.1 M NaCl, 0.1 M phosphate, 2 mM inositol hexaphosphate, 2 mM 2,3-diphosphoglycerate, all at pH 7.4.
‡ The ratio of these "R" and "T" affinities also varied as a function of conditions from around 2 to 16.

The cobalt porphyrins (Table 1) provide strong confirmation of the simple nature of O_2 binding. The models with an unhindered imidazole (i.e., NMeIm) have $P_{1/2}$ values virtually identical to those of the reconstituted cobalt hemoproteins (Table 3). In addition, the $\Delta H°$ and $\Delta S°$ of CoMb also compare well: −12.8 ± 0.6 kcal/mol and −39 ± 1 eu for the models compared to a range of −13.3 to −11.3 kcal/mol for $\Delta H°$ and −40 to −33 eu for $\Delta S°$ (24, 25) (standard state 1 atmosphere). In line with these $\Delta S°$ values is that predicted on statistical mechanical grounds (19) due to the loss of translational and rotational entropy of bound O_2. A note should be made in comparing these studies with previous cobalt porphyrins.

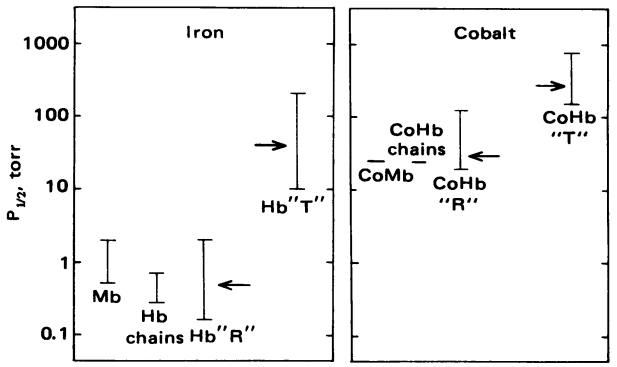


FIG. 2. Graphic compilation of O_2 affinities of hemoproteins and "picket fence" porphyrins. Model porphyrin affinities are shown by arrows; full numerical data appear in Tables 1, 2, and 3.

Earlier measurements of $P_{1/2}$ of simple cobalt porphyrins such as cobalt protoporphyrin IX dimethyl ester-(NMeIm) (29), cobalt(*p*-methoxyphenyl)porphyrin-(NMeIm) (9, 30), and cobalt tetratolylporphyrins with appended bases (31) showed very low affinities, 300 times worse than our models or the hemoproteins. This appears to be due to a selective solvation of the simple flat porphyrins favoring their deoxy form. A full discussion has already been made (14).

A major point contained in Tables 2 and 3 concerns the T state of Hb and CoHb. Within the confines of the Hoard–Perutz stereochemical mechanism for cooperativity (1), Hb is viewed as having two alternative quaternary structures: the liganded, R state, whose O_2 affinity is essentially that of the isolated subunits, and the deoxy, T state, whose O_2 affinity is diminished. The lessened affinity of the T state is presumed to be caused by *constraint* of the proximal histidine to its unliganded equilibrium position in which the Fe is ~0.5 Å out of the mean porphyrin plane. Upon ligation of O_2 (or other small molecules) to the T state form, a tension develops as the iron atom moves into the mean porphyrin plane, attempting to drag the constrained proximal histidine with it. This T quaternary state is stabilized by direct salt bridges and hydrophobic contacts between the subunits, as well as by indirect bonds between them mediated by solvent ions.

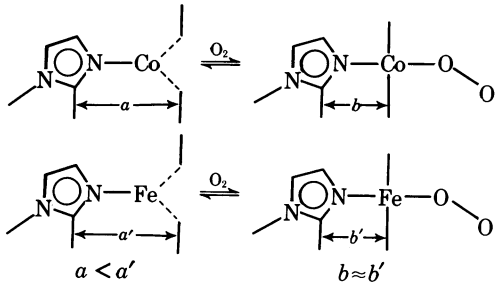
In order to probe the nature of this steric restraint experimentally, we have turned to sterically hindered axial bases. In the sense that the 2-methyl group of Me_2Im provides restraint to the motion of this axial base towards the porphyrin upon oxygenation, FeTPivPP(Me_2Im) and CoTPivPP(Me_2Im) are models for the T form of Hb and CoHb. The presence of such steric restraint with Me_2Im is obvious from the existence of 5-coordinate ferrous porphyrins (as noted earlier, with unhindered imidazoles $K_3 > K_1$) and is confirmed by crystal structures of Fe tetraphenylporphyrin(2-methylimidazole) (32), FeTPivPP(2-methylimidazole) (with J. A. Ibers, unpublished data), and Co tetraphenylporphyrin(Me_2Im) (33).

It is interesting that the hindrance offered by the 2-methyl group of Me_2Im is just that required to reduce O_2 affinities to the level found in T state Hb and CoHb (Tables 2 and 3). In the iron systems, the $\Delta\Delta G°$ (the difference in O_2 affinities of metalloporphyrins with NMeIm and Me_2Im) of our model systems is, at 25°, 2.5 kcal/mol, which is to be compared to $\Delta G°_{41}$ of Hb (the free energy difference between the first and fourth intrinsic O_2 affinities); $\Delta G°_{41}$ for Hb at 25° ranges from 2.1 to 3.7 kcal/mol, depending on conditions (23). In the cobalt systems, $\Delta\Delta G°$ of the model compounds is, at 15°, 0.8 kcal/mol, and $\Delta G°_{41}$ for CoHb, at 15°, is 0.4–1.6 kcal/mol, depending on conditions (23). This correspondence is happenstance, since more highly restraining bases, such as 2-isopropylimidazole or 1,2,4,5-tetramethylimidazole, should show even lower O_2 affinities. In drawing comparisons between Hb and model compounds, it is important that $\Delta G°_{41}$ or, equivalently, the intrinsic equilibrium constants of Hb (as differentiated from the Adair constants, which need to be corrected for the statistics of the situation) (27) be used.

The stepwise O_2 affinities of Hb shown in Table 2 have been reported by a large number of researchers (23, 34–37). The data used in this paper, those of Imai *et al.* (23), were chosen for the range of conditions and the comparable CoHb data. Generally, the data from these various groups agree well.

From Table 1, it can be noted that the restraint induced by Me_2Im in the iron porphyrins is reflected in the enthalpy of O_2 binding, the entropy remaining essentially unchanged. For the cobalt analogues the differences are much smaller, but still appear to be enthalpic. This is as one would expect, since the $\Delta S°$ of binding is determined (19) almost exclusively by the loss of translational and rotational entropy of the O_2.

Before this work was completed, we made the prediction (14) that for the same hindered bases, FeTPivPP should show a much greater change in O_2 affinity compared to an unhindered base than would CoTPivPP. Since the metal atom is further out of the mean porphyrin plane for five-coordinate iron(II) than cobalt(II) (32, 33, 38), one expects a greater change in the steric interaction of a bound Me_2Im for the iron(II) than cobalt(II) upon oxygenation, as illustrated below. That is to say, more



steric interaction between the hindered axial base and the porphyrin has *already* developed in the deoxy form of CoTPivPP than FeTPivPP, decreasing the stability of MTPivPP(Me_2Im) relative to MTPivPP(NMeIm) more for M = Co than Fe. Hence, we expect a greater decrease in O_2 affinity for FeTPivPP (B) than for CoTPivPP(B) in changing B from NMeIm to Me_2Im. This is completely analogous to the lessened cooperativity shown by CoHb relative to native Hb. Exactly this phenomenon is observed.

This same reasoning argues that even if T state deoxyHb is restrained to its normal deoxy geometry (and hence unstrained) (1), then T state deoxyCoHb *must* be strained relative to R state deoxyCoHb or deoxyCoMb.

CONCLUSIONS

By use of a series of iron and cobalt "picket fence" porphyrins, we have been able to reproduce the O_2 affinities of Mb, Hb, CoMb, and CoHb. The basic O_2 affinity shown by model ferrous and cobaltous porphyrins is the same as that of unrestrained hemoproteins (e.g., Mb, isolated chain Hb, R state Hb); special interactions between the protein and the bound oxygen are not needed to explain these hemoproteins' oxygen affinities in contrast to those for carbon monoxide (28).

By tailoring the steric interactions between the axial imidazole and the porphyrin we can mimic the decrease in ligand affinity shown in T state Hb. The restraint presumed present in the T form of Hb and CoHb has been well modeled by FeTPivPP(Me_2Im) and CoTPivPP(Me_2Im), and provides evidence *on a molecular level* that the Hoard–Perutz mechanism is viable.

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