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Cooperativity in O_2 binding to iron porphyrins

(hemoglobin/ferrous hemes/myoglobin/"picket fence" metalloporphyrinates)

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ABSTRACT **The solid–gas O_2 binding equilibrium has been studied for ferrous "picket fence" porphyrinates with sterically hindered axial imidazoles. Such systems show significant cooperativity in their binding of O_2: at low O_2 pressures a low O_2 affinity form exists, and at high O_2 pressures a higher O_2 affinity form develops. Direct analogies are drawn to the cooperativity shown in O_2 binding by hemoglobin. These model systems mimic hemoglobin quantitatively.**

The nature of oxygen binding to hemoproteins has been the subject of intense research (1–3). Of particular interest is the structure and function of hemoglobin (Hb). Hb is essential to O_2 transport in all vertebrates. It is a tetrameric protein, each subunit of which is composed of a polypeptide of ~140 residues and a prosthetic iron porphyrin held in place by iron coordination to the imidazole of a histidine residue and by ~35 nonpolar contacts at the periphery of the porphyrin. Hemoglobin's most interesting property, however, is its cooperative binding of O_2 and other ligands. This ability to show high affinity at high partial pressures of O_2 (e.g., in the lungs) and low affinity at low O_2 pressures (e.g., in the tissues) is critical to efficient O_2 transport. We report here the quantitative modeling of such cooperativity in simple ferrous porphyrins.

MATERIALS AND METHODS

All experimental operations requiring an inert atmosphere were carried out in a Vacuum Atmosphere Co. Dri-Lab under nitrogen. Magnetic susceptibilities were determined by the Faraday method, as previously described (4). Elemental analyses were determined by the Stanford Microanalytical Laboratory.

All solvents were distilled and stored under N_2. Toluene was distilled from Na metal, ethanol from Mg. 1,2-Dimethylimidazole (Me_2Im) was distilled under vacuum from Na metal, and 2-methylimidazole (2MeIm) was recrystallized several times from hot benzene.

***Meso*-tetra($\alpha,\alpha,\alpha,\alpha$-*o*-pivalamidophenyl)porphyrinato Iron(II)-2-methylimidazole [FeTpivPP(2MeIm)].** (Fig. 1.) A solution (4) of FeTpivPP (500 mg) and 2MeIm (200 mg) in 70 ml toluene was refluxed under N_2 atmosphere. Vacuum evaporation to 25 ml yielded a powdered precipitate. To this, 150 ml ethanol was then added with heating, and the product was recrystallized from this hot solution. The visible spectrum was typical of five-coordinate ferrous tetraphenylporphyrins: λ_{max} at 370, 436, 540, 558, and 600 nm in toluene. Magnetic moment under Ar was 5.1 Bohr magnetons, confirming the sample to be a high spin ferrous porphyrin. Under air, the magnetic moment dropped to less than 1.5 Bohr magnetons, demonstrating the porosity to and binding of O_2 by the sample; this was reversible upon evacuation. Elemental analyses were performed under air. Calculated for O_2 FeTpivPP(2MeIm)·C_2H_5OH: C, 68.6; N, 11.43; H, 6.25; Fe, 4.56; found: C, 68.4; N, 11.56; H, 6.21; Fe, 4.52. Solvate ethanol was confirmed by vapor phase chromatography. Single crystals have been grown and full x-ray crystal structure analysis has been accomplished, confirming the above chemical analysis and structure (5). The solvate ethanol was volatile and was lost upon heating or evacuation, as confirmed by elemental analysis and vapor phase chromatography.

***Meso*-tetra($\alpha,\alpha,\alpha,\alpha$-*o*-pivalamidophenyl)porphyrinato Iron(II)-1,2-dimethylimidazole [FeTpivPP(Me_2Im)]** (Fig. 1). A solution of FeTpivPP[4] (150 mg) and Me_2Im (60 mg) in 20 ml toluene was refluxed under N_2 and 20 ml ethanol was added; product crystallized from this solution upon cooling. The visible spectrum was typical of five-coordinate ferrous tetraphenylporphyrins: λ_{max} ($\log_{10}\varepsilon$, in M^{-1} cm^{-1}) at 371 nm (4.48), 437 (5.31), 538 (3.79), 559 (3.87), 600 (31.5) in toluene. Magnetic moment under Ar was 5.9 Bohr magnetons; under air, <1.4 Bohr magnetons. Elemental analyses were performed under air. Calculated for O_2 FeTpivPP(Me_2Im): C, 69.4; N, 11.71; H, 6.08; Fe, 4.68; found: C, 69.2;.N, 11.4; H, 6.16; Fe, 4.70. A portion of FeTpivPP(Me_2Im) was exposed to an atmosphere of CO. Elemental analysis calculated for CO FeTpivPP(Me_2Im): C, 70.7; N, 11.8; H, 6.10; Fe, 4.69; found: C, 70.6; N, 11.6; H, 6.03; Fe, 4.71.

Solid-State Oxygen Equilibria. The simple manometric adsorption apparatus used to determine oxygen adsorption isotherms for these solid samples was essentially the same as that previously described, (6, 7) consisting of a sample volume connected to a vacuum manifold and to a manometer by a three-way stopcock. The electronic manometer, a Datametrics, Inc., Barocel model 1174-570 with ranges 0–1, 0–10, 0–100, and 0–1000 torr (1 torr = 133 Pa) with a 4½-digit readout, was equipped with a thermostatted base, and was well insulated and maintained at a constant temperature throughout the experiments. The temperature of the remainder of the apparatus was controlled to better than ±0.1° with a constant temperature bath. Volumes were calibrated by expanding nitrogen from an outside gas bulb of known volume, temperature, and pressure into the evacuated apparatus. Total volume of the apparatus was ~15 ml and sample sizes were ~50 mg. Sample deoxygenation was effected by evacuating for several hours at 1 μtorr and ~50°. Oxygen at a known pressure was then expanded from the manometer volume into the sample volume at the chosen temperature. Observed pressure drops provided the moles of O_2 adsorbed by the sample as a function of O_2 pressure.

Abbreviations: Hb, hemoglobin; Mb, myoglobin; Me_2Im, 1,2-dimethylimidazole; NMeIm, 1-methylimidazole; 2MeIm, 2-methylimidazole; TpivPP, *meso*-tetra($\alpha,\alpha,\alpha,\alpha$-*o*-pivalamidophenyl)porphyrinate; $P_{1/2}$, O_2 pressure at half saturation; y, fractional coverage, fractions of sites binding O_2.

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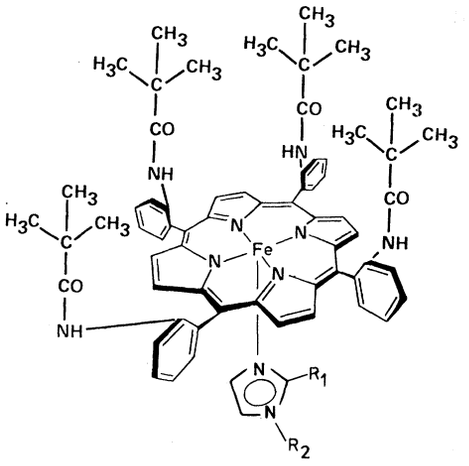

FeTpivPP(NMeIm): $R_1 = H$, $R_2 = CH_3$
FeTpivPP(2MeIm): $R_1 = CH_3$, $R_2 = H$
FeTpivPP(Me_2Im): $R_1 = R_2 = CH_3$

FIG. 1. The "picket fence" ferrous porphyrins.

RESULTS

O_2 binding in the solid state was recorded over 5 orders of magnitude in oxygen pressure for FeTpivPP(2MeIm) and FeTpivPP(Me_2Im). These data are presented in Fig. 2. The striking result, in both of these samples, is the cooperativity shown in O_2 binding, reminiscent of Hb. In both cases, the stoichiometry of O_2 binding was established to be one O_2 molecule per Fe. In the high O_2 pressure regime, the moles of O_2 adsorbed for each sample equalled the weighed amount of iron porphyrin to within 1%. Complete reversibility has been

Table 1. Cooperativity in O_2 binding, 25°

System	$P_{1/2}$, torr: High affinity "R"	$P_{1/2}$, torr: Low affinity "T"	Hill coefficient
Fe(TpivPP)(NMeIm)	0.5 (noncooperative)		1.0
Fe(TpivPP)(2MeIm)	0.7	14	2.6
Fe(TpivPP)(Me_2Im)	14	112	3.0
Hb A, stripped, pH 7.4*	0.3	9	2.5

* Refs. 8 and 9.

observed with each sample: data were collected at random over the range of O_2 pressures used, and even after >50 cycles between O_2 and vacuum, no discernible change had occurred in the O_2 binding by the sample (to within experimental error, <0.5% of moles of O_2 adsorbed). A fresh sample of FeTpivPP(2MeIm) was prepared, completely independently from any materials used in the first sample; the O_2 binding curve of this second sample was superimposable on that of the first. In order to demonstrate that these experiments were free of systematic error, a sample of FeTpivPP(NMeIm) was examined in the same apparatus; this porphyrin had been previously studied (5) with another apparatus and found to be noncooperative in its O_2 binding in the solid state. Such remained the case in our present study: over the range of 20–99% oxygenation, no deviation from simple noncooperative, Langmuir behavior occurred, and the measured pressure at half saturation, $P_{1/2}$, agreed well with the previous work.

Table 1 collects the O_2 binding equilibria in numerical form. High and low O_2 affinities were taken in the usual fashion (10, 11) from the linear unit slope regions of the Hill plots of Fig. 2 at high and low pressure, respectively. Fig. 3 presents the Hill

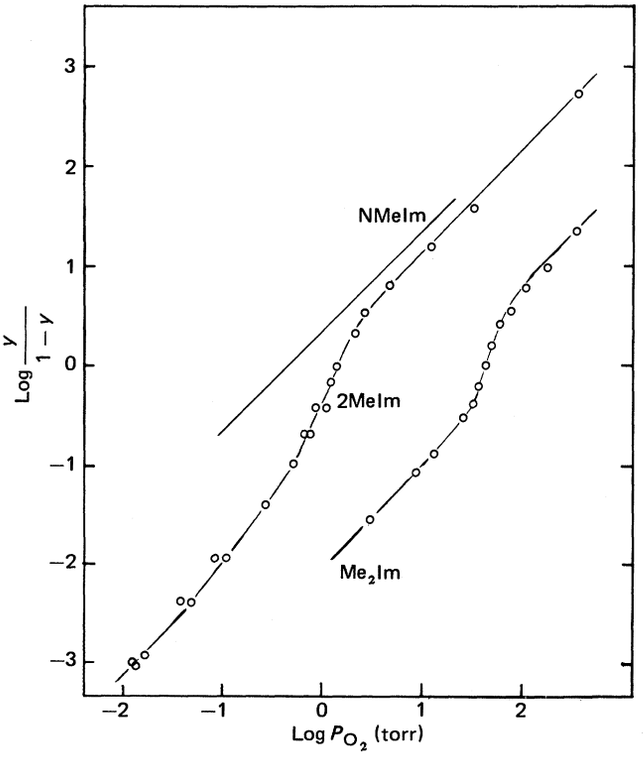

FIG. 2. Cooperativity in solid state O_2 binding by picket fence ferrous porphyrins. As labeled, the Hill plots refer to FeTpivPP(NMeIm), FeTpivPP(2MeIm), and FeTpivPP(Me_2Im), all at 25.0°.

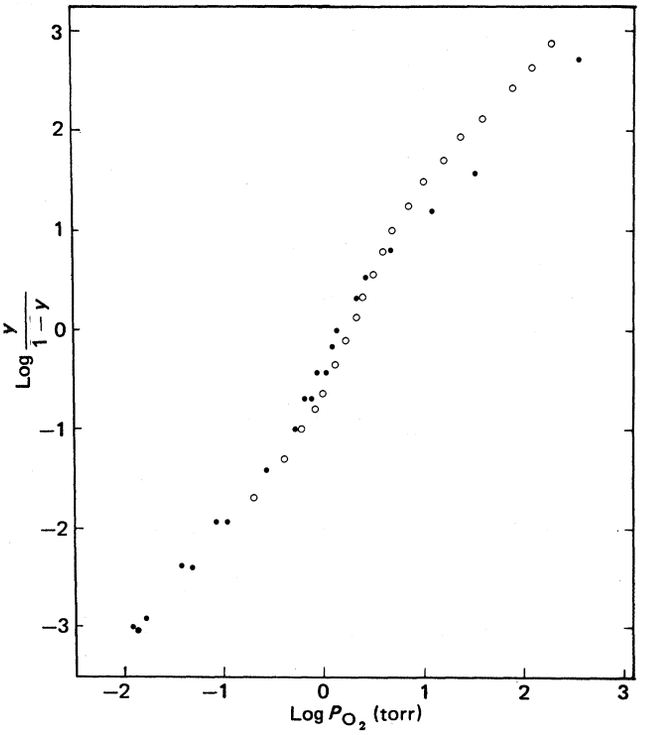

FIG. 3. Cooperativity in O_2 binding: a comparison between Hb A (○) and FeTpivPP(2MeIm) (●) at 25.0°. Hb A is in 0.05M bis-tris buffer, pH 7.4. FeTpivPP(2MeIm) is in the solid state.

plots of solid FeTpivPP(2MeIm) and Hb A at pH 7.4 in 0.05 M bis-tris [bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane] buffer (8, 9).

DISCUSSION

As can be seen in the Hill plots of Fig. 2, the picket fence porphyrins with hindered axial bases show cooperativity in their solid state O_2 binding: there exist two regimes of noncooperative binding (at high and low O_2 pressures) and an intermediate region of cooperative binding. From the binding at high and low O_2 pressure, one can extrapolate the $P_{1/2}$ values of the high and low affinity forms, respectively. These are presented in Table 1, along with similar values for human Hb (8, 9).

Hb shows rather wide variations in its low affinity (T state) O_2 binding equilibrium constants, depending on pH, Cl^- concentration, and phosphate concentration (9–11). This is due to the ability of the low O_2 affinity (T) conformation to bind ions much more strongly than the high O_2 affinity (R) form (1). In this way, addition of ionic species to a Hb solution stabilizes the deoxy T form selectively and decreases its O_2 affinity. Our model systems, of course, do not have this characteristic, and so comparisons are best drawn between our models and Hb stripped of interfering ions, as in Table 1. The resemblance is quite striking; the strong similarity between the picket fence porphyrins and Hb A is especially apparent for FeTpivPP(2MeIm). This is graphically demonstrated in Fig. 3: the O_2 binding affinities of our model system and of stripped Hb are extremely close.

The exact molecular mechanism of the cooperativity in our models requires further investigation. Our current hypothesis invokes a slight change of the molecular dimensions upon oxygenation. Five-coordinate, deoxy ferrous porphyrins are high spin, with the iron atom out of the mean porphyrin plane by ~0.5 Å. Upon oxygenation, the spin state becomes low spin and the iron moves into the mean porphyrin plane, (1, 3) thus shrinking the overall dimension of the FeTpivPP(2MeIm) molecules, for example, by ~0.6 Å or ~5%. The extended regions of unit slope in Fig. 1 imply two well-defined forms, one at high O_2 pressure and one at low, each of which persists over its own wide range of pressure and coverage. The steric hindrance provided by the 2MeIm or Me_2Im decreases the O_2 affinity of the deoxy, five-coordinate iron (12). As molecules in the solid oxygenate, the change in molecular dimensions presumably induces strain in the crystallite. Eventually, this strain must be sufficient to induce a conformational change in the solid which enhances the O_2 affinity of the remaining deoxy sites. This may be analogous to the Hoard–Perutz mechanism of Hb cooperativity (1). The striking similarity in the Hill plots of our models and of Hb, as in Fig. 3, is dependent on choice of axial base and should not be given exaggerated importance. Nonetheless, the match between the ratio of high and low affinities of stripped Hb and our models may prove to be significant. Having neither protein subunits nor intersubunit contacts, these models cannot pretend to explicate those portions of Hb cooperativity. However, the active sites of Hb and of these solids are quite analogous (both are high spin, five-coordinate, ferrous porphyrin–imidazole systems capable of binding O_2, CO, etc.), and it is reasonable that the effects that cause the cooperativity seen in both systems are likely to be similar at the metal site. The differences, protoporphyrinate IX instead of TpivPP, protein subunits instead of crystals, intersubunit contacts instead of crystal packing forces, should not, of course, be ignored. In general, the cooperativity of Hb arises from indirect heme–heme interactions, whereas the cooperativity of our picket fence porphyrins must arise from more direct interactions.

Crystal structures of FeTpivPP(2MeIm)·EtOH and its O_2 adduct have been completed and will be published elsewhere (J. P. Collman, J. A. Ibers, G. Jameson, F. S. Molinaro, E. Rose, and K. S. Suslick); this material has the same space group (C^6_{2h} – $C_{2/c}$) and the same basic unit cell structure (with four molecules per asymmetric unit) as the noncooperative (O_2)FeTpivPP(NMeIm) (4, 5, 12).

The decreased affinity of FeTpivPP(Me_2Im) relative to FeTpivPP(2MeIm) is well explained by the buttressing effect in Me_2Im. The interaction of the 1-methyl group with the 2-methyl group will increase the steric contact between the 2-methyl group of Me_2Im with the porphyrin plane, relative to 2MeIm. The slightly greater hindrance is sufficient to decrease further the O_2 affinity of the metalloporphyrin.

Solution data are available for O_2 binding to FeTpivPP(Me_2Im) (13) and are consistent with the proposed mechanism of cooperativity. The observed $P_{1/2}$ at 25° in toluene is 38 torr (no cooperativity is observed in solution), which falls midway between the high and low affinities of the cooperative solid. This equilibrium constant may include a slight solvation effect, due to selective solvation of the deoxy or oxy form, but in our studies with picket fence porphyrins these effects are relatively minor (less than a factor of 2 in $P_{1/2}$) (6, 13).

There are two previous reports in the literature of cooperative O_2 binding to synthetic iron porphyrins. The interesting work of Tsuchida's group with ferrous hemes and poly(L-lysine) shows a "pseudo-cooperativity" in binding both O_2 and CO (14–16). These workers have clearly demonstrated that the origin of this effect in their systems is the competition between O_2 and a *second* amine residue of the poly(L-lysine) to bind to a putative five-coordinate ferrous heme–poly(L-lysine) complex. In this polymer, as O_2 is bound it displaces the *second* amine residue and uncoils the α helix of the poly(L-lysine), thus diminishing the stability of the remaining ferrous heme–bis(amine) (i.e. deoxy) complexes and hence increasing their tendency to bind O_2. This pseudo-cooperativity, then, is of a much different sort than that of Hb or our present models: Tsuchida's models involve competition in the formation of the oxygen complex from a bis(axial base) complex; Hb (and our models) involves only the five-coordinate heme complex and its O_2 adduct. In Tsuchida's work with O_2, excess dithionite is present to reduce the ferric porphyrins formed and hence oxygenation *appears* reversible; such is not the case with our sterically hindered picket fence porphyrins, which show true reversibility. The brief report of cooperative O_2 binding in another polymeric system by Bayer (17) is much less clear. The "reduced" deoxy visible spectra reported match the typical hemochrome spectra of ferrous protoporphyrin IX bis(imidazole), bis(pyridine), or nearly any six-coordinate, low spin ferrous complex (18–20). Hence, Bayer's polymer is likely to be akin to Tsuchida's. However, the visible spectrum of the O_2 adduct reported by Bayer does not correspond to that of known dioxygen complexes (8, 18–20) (such as oxy Mb and Hb) with closely similar hemes. Finally, Bayer's O_2 binding isotherm, when displayed as a Hill plot, is not well behaved, having no regime of unit slope, in contrast to Hb, Tsuchida's system, and our own. The exact nature of the equilibria involved in Bayer's report thus remains in doubt.

CONCLUSION

Two synthetic porphyrin systems have been presented, which model in a quantitative fashion the cooperativity of Hb. In spite of the absence of protein subunits and subunit contacts in our compounds, similar O_2 binding behavior occurs for Hb and our

models. The origin of the cooperativity seen in our picket fence porphyrins is apparently the change of the molecular dimension of the porphyrins upon oxygenation, communicated from site to site by the crystal packing forces. Crystal structures are in final stages of refinement and will be reported shortly. In contrast to previous model studies, the cooperativity that our porphyrin complexes exhibit is analogous to that of Hb in mechanistic and quantitative detail.

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