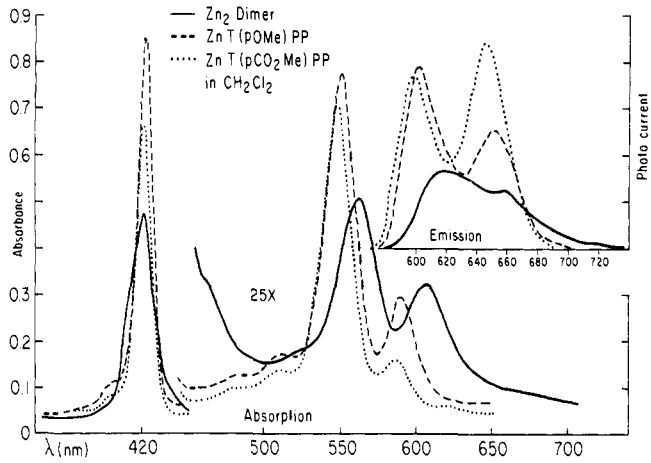

Figure 3. Absorption spectra of 1.2-μM and 30.0-μM solutions of the zinc metalloporphyrins in CH_2Cl_2 with the baseline offset from zero for clarity. Uncorrected emission spectra of equally absorbant solutions at 420 nm were excited at this wavelength. Homogeneity was verified by excitation-emission spectral analysis.

ticipate that the method will be general and that the correlation of structures with properties will significantly aid the interpretation of in vivo and in vitro porphyrin systems such as the photosynthetic chlorophyll dimers. The effects of systematic changes in ring separation, orientation, donor-acceptor substituents, metal and mixed-metal chelates, and intercalates can now be examined.

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(13) Contains traces of chlorin (Figure 2) which were not removed since final condensation to III would re-form chlorin in both old and new rings via autoxidation-reduction; cf. D. Dolphin, *J. Heterocycl. Chem.*, **7**, 275 (1970).
(14) Alumina was deactivated by saturation with ethyl acetate followed by drying at 115 °C for 1 h.
(15) Assignments are consistent with spectra of T(*p*-OCH_3)PP, T(*p*-CO_2CH_3)PP, and T(*p*-$CO_2CH_2CH_2OC_6H_5$)PP.
(16) (a) Low carbon analyses (here 1.2%) occur in large aromatic heterocycles such as big tetraarylporphyrins despite long burning (5'), high temperatures (975 °C), and added catalyst (V_2O_5). (b) Yields were determined spectroscopically after hydrolysis (*19,20*).
(17) This 1% cross-linked polystyrene support in pyridine was chosen to minimize adsorption of aromatic elutants. Standardization of the system with synthetic porphyrins and azulenes showed the retention constant (*R*, void volume/elution volume) to be proportional to species diffusional molecular size and not molecular weight; cf. T. C. Laurent and J. Killander, *J. Chromatogr.*, **14**, 317 (1964).
(18) (a) Alternately, III may be purified by chromatography of the $CHCl_3$-soluble fraction on deactivated alumina followed by silica gel TLC with CH_2Cl_2 and 1% EtOH. Treatment of III with dichlorodicyanobenzoquinone prior to TLC converts trace chlorins back to porphyrins; cf. G. H. Barrett, M. F. Hudson, and K. M. Smith, *Tetrahedron Lett.*, 2887 (1973).
(19) Samples were hydrolyzed in propionic acid–hydrochloric acid (12 N) (1:1) at 130 °C for 1 h in sealed evacuated ampules. Products were separated either by partitioning between aqueous base and 1-butanol, or by TLC on silica gel with 2,6-lutidine/water in NH_3 vapors; cf. J. Jensen, *J. Chromatogr.*, **10**, 236 (1963). Standards were recovered in 95% yields after workups. Products were identified by their comigration with authentic samples in two TLC solvent systems, similar acid–base partitioning, and absorption and emission spectra.
(20) Extinction coefficients for III and its zinc chelate were deduced from quantitative analysis of hydrolysis products I and IV based on their known εs. This procedure was verified by hydrolysis of I, II, T(*p*-CO_2CH_3)PP, and T(*p*-$CO_2CH_2CH_2OC_6H_5$)PP.
(21) The least strained CPK structures of III require interdigitation of the phenyl rings. This gives a slight twist to the molecule and results in a limited rotation-barrier chirality.
(22) Of the several possible H_4-dimer tautomers, III (Figure 1) with diagonally staggered inner hydrogens would be most probable. This would lead to minimal interaction of the orthogonal local dipoles.
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Detection and Characterization of the Long-Postulated Fe–OO–Fe Intermediate in the Autoxidation of Ferrous Porphyrins

Sir:

Dioxygen bridged diiron complexes have been frequently proposed as important, although unstable, intermediates in the autoxidation of ferrous complexes.[1-3] Such bridged species have also been proposed to represent the oxygenated state of the respiratory pigment, hemerythryn.[4] In the development of strategies for the synthesis of low molecular weight models of myoglobins and hemoglobins, steric protection of the iron site has been deemed useful to prevent autoxidation via an Fe-OO-Fe intermediate.[5-8] Nevertheless, and in contrast to the situation with other metals, notably cobalt,[9,10] no Fe-OO-Fe complex has been unambiguously identified and none of its structural and electronic properties or chemical reactivity characterized. Only a few reports of O_2 uptake with a Fe:O_2 ratio of 2:1 have appeared which are suggestive of a Fe-OO-Fe species.[11,12] The proposed form for this bridged species has been variously described as Fe^{II}-O_2-Fe^{II} (dioxygen bridge[2,11]) or Fe^{III}-OO-Fe^{III} (peroxo bridge[2]), while others have anticipated rapid cleavage to two Fe-O monomers.[5] We report here on the ^1H NMR characterization of such a Fe-OO-Fe complex and show that the linkage involves the peroxo bridge.

As an initial step in elucidating the general mechanism of oxidation of ferrous porphyrins, we have investigated the reaction between unligated *meso*-tetra-(*m*-tolylporphyrin)-iron(II), designated PFe, and molecular oxygen in dry toluene-d_8. The final product is the expected μ-oxo dimer, PFeOFeP, and the proposed steps thought to account for the net reaction can be written

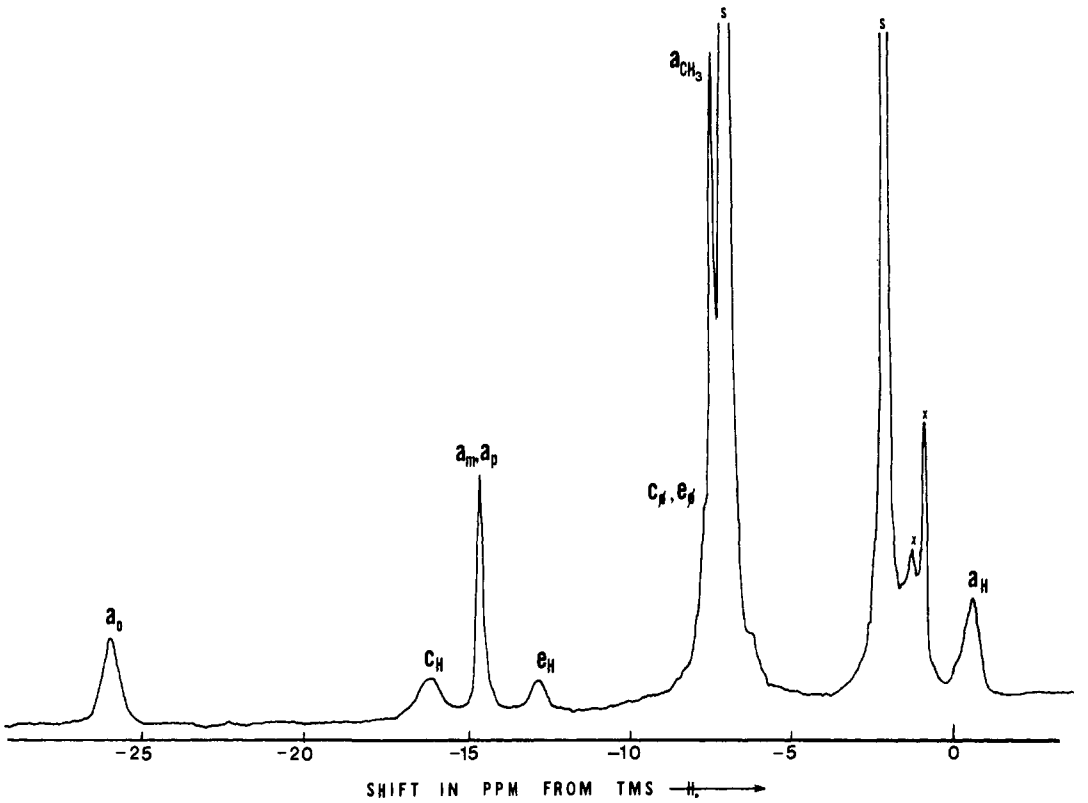

Figure 1. Proton trace of sample containing PFe (A), whose peaks are labeled a_i, PFe-OO-FeP (C), with peaks designated c_i, and PFe-O-FeP (E) with peaks marked e_i, in toluene-d_8 at −50 °C. The subscript to the labels, o, m, and p, refer to ortho H, meta H, and para H, ϕ to the unresolved composite of ortho, meta, and para H, CH_3 to *m*-CH_3, and H to the pyrrole H; S = solvent peaks and X = impurities.

$$\underset{A}{PFe} \xrightarrow[1]{O_2} \underset{B}{PFeO_2}$$

$$\xrightarrow[2]{PFe} \underset{C}{PFe\text{-}OO\text{-}FeP} \xrightarrow[3]{} \underset{D}{PFeO} \xrightarrow[4]{PFe} \underset{E}{PFeOFeP} \qquad (5)$$

The highly characteristic ^1H NMR shifts[13] of iron porphyrins in various oxidation and spin states suggested to us the possibility of identifying and characterizing some of these proposed intermediates.

A 5 mM solution of PFe in degassed, dry toluene-d_8 at −50 °C yields the previously assigned[14] proton trace whose peaks are labeled a in Figure 1. Introduction of some dry O_2 results in the appearance of a new set of peaks labeled c, which are assigned to the dimer C. Apparently B rapidly reacts with A to yield C. The pyrrole-H assignment for C is verified by spectra of an FeP bearing perdeuterated phenyl groups.[14] Raising the temperature to −30 °C for a few minutes and again cooling to −50 °C indicates that some C has been converted to E (peaks labeled e in Figure 1), which is readily identified as the previously characterized oxo dimer.[15] Solutions containing only C can be prepared. These solutions are stable indefinitely at −80 °C (≥2 weeks) and for ~1 h at −30 °C. Repeated freeze-pump-thaw cycles at −80 °C indicate that the step A → C is irreversible.[16]

Species A, C, and E are also readily distinguished by their electronic spectra. The most characteristic differences occur in the 480-650-nm region. In toluene solution at −80 °C prominent absorption maxima for these species are as follows: A, 536 nm (ϵ 3 × 10^4); C, 630 (5 × 10^3), 540 (2 × 10^4), 480 (2 × 10^4); E, 606 (1 × 10^4), 565 (2 × 10^4).

When a solution containing both A and C is purged of any dissolved oxygen and warmed anaerobically to −30 °C, conversion of a unit of C → E is accompanied by simultaneous conversion of two units of A → E, as detected by the integration of the A and C pyrrole-H peaks before warming and the A, C, and E pyrrole-H peaks after warming to −30 °C.[17] Thus C must possess at least one O_2 per two Fe in the complex, which is consistent with the Fe-OO-Fe structure. A dimeric structure is independently indicated by noting that, when O_2 is added to a −80 °C toluene-d_8 solution containing both PFe and P′Fe (P′ = octaethylporphyrin[14]), *two* pyrrole-H C peaks are observed, one for PFe-OO-FeP and one for PFe-OO-FeP′.

Additional proof for the dimeric nature of C and a characterization of its electronic structure result from solution susceptibility measurements and the temperature dependence of the pyrrole-H contact shift. At ~−50 °C, the magnetic moments for A, C, and E were found to be 4.5, 2.6, and 1.5 μ_B, respectively; μ for both A and E correspond closely to published values.[14,15,18,19] While μ for A remains constant, that at C decreases to 2.2 μ_B at −83 °C. Hence, C, as previously shown for E,[14] is antiferromagnetic, and a peroxo formalism, i.e., Fe^{III}-OO-Fe^{III} is strongly indicated. A frozen toluene glass of C at 77 K failed to yield an ESR spectrum. The larger magnetic moment of C relative to E, when interpreted by a coupling between two high spin ferric ions,[20] indicates an antiferromagnetic coupling 2 J ~265 K, which is ~30% smaller than that for PFe-O-FeP.[19]

The weaker coupling of the ferric ion in C compared to E is also supported by the pyrrole-H contact shifts. The decrease in shift at lower temperature reflects the antiferromagnetism,[14] and the percent decrease in the shift can be taken as a measure of the strength of the coupling. Figure 2 shows that the percent decrease in pyrrole-H contact shift for PFe-OO-FeP is smaller than that for PFe-O-FeP. The weaker coupling in the former species can be attributed to its longer bridge.

Current and future work is directed at detecting B and D in solution, as well as determining the effect of a nitrogeneous axial base on the stability and electronic structure of the various intermediates. The role of the axial base[21] may control the

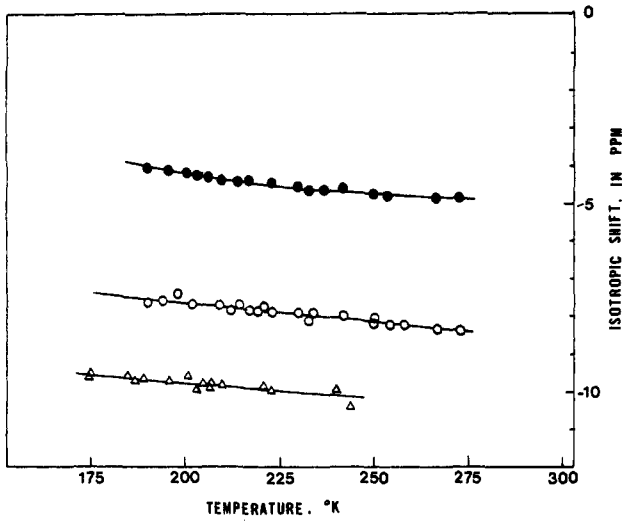

Figure 2. Graph of pyrrole-H isotropic shift vs. temperature for PFe-O-FeP, ●; PFe-OO-FeP, ○; and PFe-OO-FeP', △. The decrease in isotropic shift on lowering the temperature 273 to 200 K is 14% for PFe-O-FeP and only 9% for PFe-OO-FeP.

stability of a dioxygen vs. a peroxo bridge and thereby determine whether the step A → C is reversible.

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^{15}N Nuclear Magnetic Resonance as a Probe of Residual Structure in the Backbone of Unfolded Hemoglobin

Sir:

An important aspect of dynamic studies of protein folding is the determination of the degree of order that exists in unfolded polypeptides.[1-3] Optical rotation, UV absorption, viscosity, enzyme activity, NMR, and Raman spectroscopic measurements[4-8] on unfolded proteins have provided information on the existence of highly ordered residual structures that contain stable side-chain–side-chain interactions. However, simpler residual structures consisting of residues whose available conformations have been restricted by short range interactions with neighboring residues have proved to be more difficult to detect.[9] NMR is a tool remarkably well suited for assaying the degree of time averaged randomness of each residue in the backbone of unfolded proteins since ^{1}H, ^{13}C, and ^{15}N chemical shifts of peptide nuclei are sensitive to conformation and solvation effects.[10] Unfortunately, the ^{1}H, ^{13}C, and ^{15}N NMR spectra protein backbones display notoriously little resolution. We here report that exchange of labile N-H hydrogens for deuterium is a convenient method for considerably improving resolution in the ^{15}N NMR spectra of protein amide groups. Using this procedure, we have been able to observe a number of glycyl ^{15}N resonances, spread over 20 ppm, in the ^{15}N NMR spectrum of [Gly-^{15}N] hemoglobin in D_2O. Upon acid and alkaline denaturation of hemoglobin and globin, not all resonances shift to the random coil position, which provides evidence that in denaturated globins there are glycyl residues, whose conformations have not been completely randomized.

A Me_2SO-treated Friend virus induced murine leukemic cell culture grown in medium containing [^{15}N]glycine (95% ^{15}N) was used to prepare hemoglobin, Hb-[Gly-^{15}N], whose glycyl residues and heme groups were labeled to 50% with ^{15}N.[11] The Friend luekemic cell hemoglobin mixture studied[11] consists of hemoglobins composed of DBA/2 mouse α^{major} globin chains, containing 11 glycyl residues at A2, A13, A16, AB1, B3, B6, D7, E7, E6, E20, and EF7, β^{major} globin chains with 14 glycyl residues at A10, A16, B4, B6, B7, CD5, D7, E8, E18, EF7, G9, G17, GH2, and H14, and β^{minor} globin chains, which lack the A16 glycyl residue.[12] This heterogeneous group of labeled glycyl resides consists of residues with a variety of ϕ-ψ values and hydrogen-bonding modes and reside in the middle and terminal regions of regular and irregular helices, in interhelical bends, as well as at the $\alpha_1\beta_1$ contact.

The proton-coupled 9.12-MHz ^{15}N NMR spectrum of carbomonoxy [^{15}N-Gly]hemoglobin (CO-Hb-[Gly-^{15}N]) displays a set of three broad, poorly resolved resonances centered at 80.9 ppm when measured at a concentration of 3.7 mM in aqueous 0.05 M, pH 7.5 phosphate buffer, which were similar to those reported previously.[11] Proton broad-band noise decoupling produces a ^{15}N spectrum of lower intensity (NOE ~ 0.25) consisting of a major resonance at 80.9 and a minor resonance at 88.2 ppm. An improvement in resolution could be obtained by dilution to 0.8 mM, which allowed the 80.9-ppm resonance to be observed as doublet with $^{1}J_{NH}$ = 95.2 ppm in the proton-coupled ^{15}N spectrum.

Exchange of the hemoglobin amide protons with deuterium at 10 °C over the course of several days had a pronounced effect on the proton coupled ^{15}N NMR spectrum (Figure 1a) of CO-Hb-[Gly-^{15}N] (0.8 mM in D_2O phosphate buffer, pD 7.5, 0.05 M). The spectrum displays no less than seven clearly resolved, narrow, and reproducible resonances spanning a chemical shift range of 20 ppm. *This spectrum demonstrates the marked improvement in the resolution of ^{15}N spectra of proteins which can be obtained simply by exchanging the labile amide hydrogen with deuterium.*