Evidence for Dimer Formation of Nitrosyl(*meso*-2,3,7,8,12,13,17,18-octaethyl-5-nitroporphyrinato)iron(II) and its Implication in the Interpretation of the Electron Spin Resonance Spectrum of the Nitrosylhaemoglobin–Salicylate System

By **Hideo Kon,*** Laboratory of Chemical Physics, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205, U.S.A.
Makoto Chikira, Chemical Research Institute of Non-Aqueous Solutions, Tohoku University, Sendai, Japan
Kevin M. Smith, Department of Chemistry, University of California at Davis, Davis, California 95616, U.S.A.

Dimer formation of the title complex in toluene has been demonstrated by an electron spin resonance study of this complex at a high concentration at 9 K, and the approximate dimer structure has been determined by spectral simulation. The observation enables us to interpret the hitherto unexplained four resonance absorptions which are known to appear in nitric oxide ligated haemoglobin (NO-haem) as a result of interaction with salicylate, on the basis of similar dimer formation of the NO-haem prosthetic groups. The event appears to take place intramolecularly by association of NO-haem molecules coming out of the haem pockets, and the complex is stabilized by the globin structure.

It is known that interaction of oxyhaemoglobin with salicylate causes enhancement of oxygen uptake in the presence of a substrate such as ascorbate.[1] In an e.s.r. study of the interaction of NO-haem † with salicylate, as a possible model system for the enhanced oxidase-like activity of oxyhaemoglobin, it was reported that added salicylate causes four new e.s.r. peaks, two on each side of the main NO-haem absorption.[2] The corresponding *g* factors observed are 2.28, 2.19, 1.93, and 1.83 respectively (see Figure 1). These

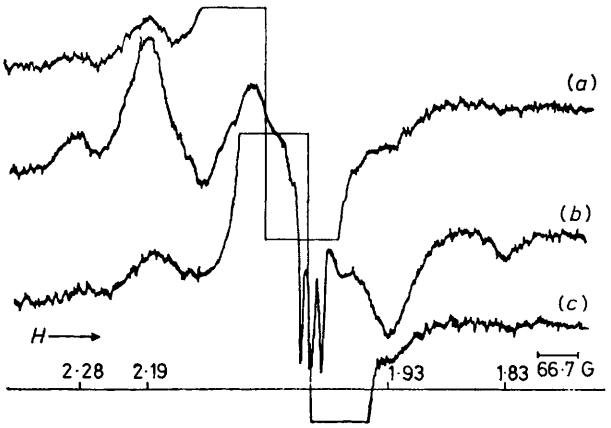

FIGURE 1 E.s.r. spectra of NO-haem–salicylate system at 77 K showing the extra peaks. The central portions of the top and bottom spectra are curtailed. Salicylate concentrations are (*a*) 1.0, (*b*) 0.1, and (*c*) 0.02 mol dm^{-3}; solvent 1×10^{-2} mol dm^{-3}, phosphate buffer pH 7; haemoglobin concentration = 4×10^{-4} mol dm^{-3} in haem (reproduced from ref. 2 by permission)

absorptions begin to appear at a salicylate concentration of *ca.* 10^{-2} mol dm^{-3}, reach their maximum intensities near 10^{-1} mol dm^{-3}, and disappear above 1 mol dm^{-3}. Meanwhile, the concomitant intensity change of the regular NO-haem signal is almost exactly opposite, namely, the lowest intensity occurring around 10^{-1} mol dm^{-3} in salicylate concentration. Thus, a part of the NO-haem species evidently undergoes some transformation as the result of salicylate perturbation in the limited concentration range of the latter, which approximately coincides with the range in which the enhanced oxygen uptake of the oxyhaemoglobin–salicylate system has been observed. The origin of these additional signals was unknown at that time, but the possibility of denatured methaemoglobin causing the extra signals was experimentally ruled out.[2]

In this report, we demonstrate, by using a model system, that dimer formation of NO-haem moieties as the result of salicylate perturbation of the globin structure is responsible for the extra e.s.r. signals.

† NO-haem = Nitrosylhaemoglobin, which contains iron(II).

EXPERIMENTAL

2,3,7,8,12,13,17,18-Octaethylporphyrin (Strem Chemicals) and *meso*-octaethyl-5-nitroporphyrin [3] were converted to the $Fe^{III}Cl$ complexes by using the iron(II) sulphate method.[4] Five-co-ordinate nitrosyl(*meso*-octaethyl-5-nitroporphyrinato)iron(II) (1) and nitrosyl(octaethylporphyrinato)-iron(II) (2) solutions in toluene for e.s.r. measurements were prepared in anaerobic apparatus attached to a high vacuum line as described previously.[5] In this apparatus, the concentration of the solution for e.s.r. can be easily changed by vacuum distillation to or from a small solvent reservoir.

Electron spin resonance measurements were made by using a Varian E 109 *X*-band spectrometer with a 23-cm magnet at 100 kHz field modulation. The microwave frequency was measured by a cavity wavemeter calibrated against the signal of an α,β-diphenyl-β-picrylhydrazyl crystal ($g = 2.003\ 6$), and the magnetic field was measured by using a proton n.m.r. probe. The temperature of the sample was held at 9 K by a Helitran system (Air Products and Chemicals Co.).

RESULTS AND DISCUSSION

An e.s.r. spectrum of (1) in frozen toluene solution (8×10^{-3} mol dm^{-3}) is shown in Figure 2. The central part of the spectrum represents a typical e.s.r. absorption of a five-co-ordinate nitrosyl complex of iron(II) porphy-

rin.[5] Examination at a higher instrument sensitivity showed that this is flanked by four satellites having g factors 2.30, 2.19, 1.93, and 1.84, which are in close agreement with those observed in NO-haem–salicylate solutions, and the observed intensity profiles are also similar (*cf.* Figure 1). These peaks lose intensity

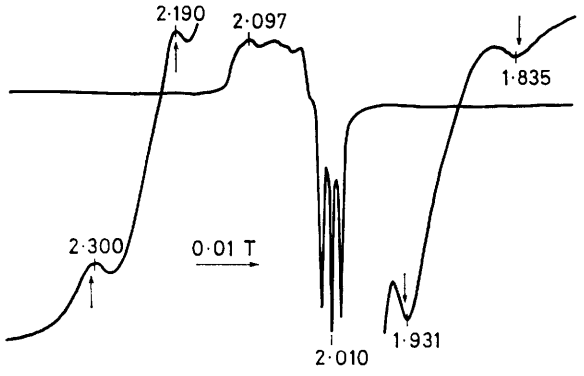

FIGURE 2 E.s.r. spectrum ($\Delta M = \pm 1$) of nitrosyl(*meso*-octaethylnitroporphyrinato)iron(II) in toluene (8×10^{-3} mol dm^{-3}) observed at 9 K. Instrument sensitivity is set *ca.* 80 times higher at the outer wings. Observed g factors are indicated by vertical bars, the calculated peak positions by arrows

on dilution, even though the spectrometer sensitivity is raised accordingly, and disappear completely at *ca.* 1×10^{-3} mol dm^{-3}. The fact that the change is reversible indicates that the satellite peaks may come from a dimer of (1) having spin exchange and dipole–dipole interaction. The satellites could not be observed in (2) even at a higher concentration. This is also in keeping with dimer formation, because the remarkable role of *meso*-nitro-substitution in enhancing dimer formation has been well demonstrated in other metal complexes of octaethylporphyrin.[6]

Crucial evidence for dimer formation is obtained by observing a half-field ($\Delta M = 2$) resonance (Figure 3), which shows a hyperfine structure consistent with electron spin interaction with two ^{14}N nuclear spins. The $\Delta M = 2$ dimer absorption is unfortunately distorted by overlapping with a large e.s.r. signal from the dimer of unreacted iron(III) *meso*-octaethylnitroporphyrin,[7,8] the formation of which apparently is much more favoured under the same conditions. Two ^{14}N nuclei with nuclear spin = 1 would give rise to five, seven, or nine hyperfine components depending upon the ratio of the two coupling constants. In the observed spectrum, a maximum of seven components can be counted but with some uncertainty in the intensity ratio due to the distorted line shape, especially of the two outermost components. In order to further confirm dimer formation, and obtain information on the geometry of the dimer, we have carried out spectral simulations based upon the following simplifying assumptions: (*i*) the principal g tensor axes of the two monomers are arranged parallel to each other (or the dimer structure has a centre of symmetry), (*ii*) the two ^{14}NO hyperfine coupling constants are of equal magnitude, and (*iii*) the interaction of the spin systems may be approximated by a point dipolar coupling.

Since both spectra of $\Delta M = \pm 1$ and $\Delta M = 2$ are superimposed on a huge background, and therefore a precise simulation of spectral lineshape is not feasible, we aimed at reproducing the observed peak positions of the first-derivative spectra using the same parameter value for $\Delta M = \pm 1$ and $\Delta M = 2$ spectra. The details of the procedure have been described previously.[9,10] The parameters to be varied are: the distance (r) between the two paramagnetic centres, and the polar (ξ) and azimuthal (η) angles which the vector r makes relative to the principal axes of the g tensor.

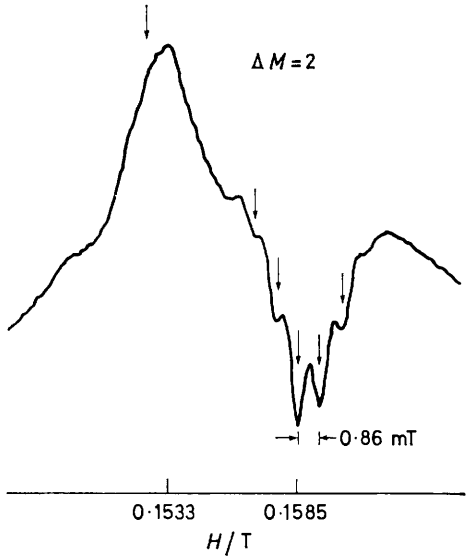

FIGURE 3 E.s.r. spectrum ($\Delta M = 2$) of nitrosyl(*meso*-octaethylnitroporphyrinato)iron(II) in toluene at 9 K. Calculated peak positions are shown by arrows

As shown in Figures 2 and 3, the calculated best-fit spectra reproduce consistently the peak positions in both $\Delta M = \pm 1$ and $\Delta M = 2$ absorptions. The largest discrepancy seen at the low-field peak of $\Delta M = 2$ may indicate the extent to which some of the basic assumptions made are not strictly adequate. For example, the spin density in the monomer is known to be distributed approximately equally between Fe and NO,[11] and consequently the point dipolar description may be an oversimplification. Nevertheless, the overall agreement of the peak positions in the calculated spectra with those in the observed spectra indicates that the structure of the dimer represented by $r = 4.3$ Å, $\xi = 40°$, and $\eta = 0°$ as obtained in the best fit should be a reasonable approximation.

If we assume that both NO groups are oriented outward in the dimer [Figure 4(*a*)], and if we place the paramagnetic centre midway between Fe and NO nitrogen, then the vertical distance (z) between the porphyrin planes is estimated to be *ca.* 1.6 Å, by using the r and ξ values obtained and a Fe–N distance of 1.717 Å.[12]

The estimated distance (z), however, is far too short

compared with known similar cases.[6,13] The two monomers are, therefore, more likely to be arranged with both NO groups in between the porphyrin planes [Figure 4(*c*)] or in head-to-tail fashion [Figure 4(*b*)], and both of the structures may actually be realised. The calculated distance (*z*), 5.0 Å and 3.3 Å respectively, for the latter two situations is more in line with the other known cases. In the head-to-tail arrangement, the two ^{14}N hyperfine coupling constants are probably unequal,

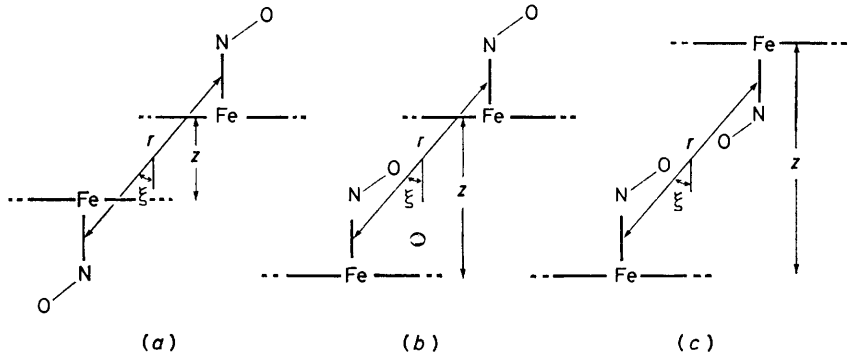

FIGURE 4 Schematic diagram of the three possible dimer arrangements. The distance between the paramagnetic centres (*r*), the skew angle (ξ), and the distance between the porphyrin planes (*z*) are indicated. The diagram is not drawn to scale, and the direction of the N–O bond is arbitrary

and there will be more than five hyperfine components. The fact that the observed hyperfine pattern (Figure 3) shows the two outermost components as having abnormally low intensity may indeed be explained by superimposing a seven-component and a five-component pattern, should these end components be taken into account.

In the model compound in toluene, the presence of a strongly electron-withdrawing NO_2 group, causing the electronic polarization between the π network and the metal ion, is a necessary condition for the dimer formation. In the NO-haem–salicylate system, where the porphyrin ring does not have a NO_2 substituent, a similar polarization effect is accomplished by the combined effect of the electrophilic vinyl and the propionate and the electron-donating methyl groups. Hydrophobic interaction between the two porphyrins in a protein environment which may be half exposed to the aqueous medium would also assist dimer formation.

In any case, there is little doubt that the four extra peaks observed in the NO-haem–salicylate system originate from dimeric species of NO-haem groups formed as a result of salicylate perturbation of the protein structure. It is interesting to note that the dimer to monomer ratio is at least two orders of magnitude greater in the NO-haem–salicylate solution than in the model system described above, even though the NO-haem concentration is much lower. Furthermore, such well defined dimer formation cannot be observed when the solution of nitrosylhaem (without globin) is prepared at various concentrations in aqueous alkaline solutions; instead, in this case, a broad featureless absorption is obtained in place of the regular monomer signal, indicating that random aggregation is taking place.

These observations mean that the dimeric species is formed from the two NO-haem complexes coming out of the haem pockets of the two adjacent sub-units, as the result of the structural perturbation of globin by salicylate. The event appears to take place before the NO-haem species are completely outside the protein, since, otherwise, polymerization would ensue. The structural perturbation by salicylate is evident from the sharp three-line components of the monomer e.s.r. spectrum (Figure 1), which has been interpreted to indicate the rupture of the bond between the F8 histidine imidazole * and the iron(II) ion.[5] A specific milieu of globin, somewhere outside the original haem pockets, seems to be required to stabilize the dimeric species.

More precise correlation of dimer formation now established in the NO-haem–salicylate system and the enhanced oxygen uptake of the oxyhaemoglobin–salicylate system requires further investigation.

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* The 8th amino-acid in the F-segment of haemoglobin.

REFERENCES

[1] G. Kikuchi and T. Tomimura, *J. Biochem.* (*Tokyo*), 1954, **39**, 503.
[2] H. Kon, *J. Biol. Chem.*, 1968, **234**, 4350.
[3] K. M. Smith, G. H. Barnett, E. Evans, and Z. Martynenko, *J. Am. Chem. Soc.*, 1979, **101**, 5953.
[4] J. H. Furhop and K. M. Smith, in ' Porphyrins and Metalloporphyrins,' ed. K. M. Smith, Elsevier, Amsterdam, 1975, p. 800.
[5] H. Kon, *Biochim. Biophys. Acta*, 1975, **379**, 103.
[6] M. Chikira, H. Kon, R. A. Hawley, and K. M. Smith, *J. Chem. Soc., Dalton Trans.*, 1979, 245, and refs. therein.
[7] M. Chikira, H. Kon, and K. M. Smith, *J. Chem. Soc., Chem. Commun.*, 1978, 906.
[8] M. Chikira, H. Kon, and K. M. Smith, *J. Chem. Soc., Dalton Trans.*, 1980, 526.
[9] M. Chikira and T. Isobe, *Bull. Chem. Soc. Jpn.*, 1972, **45**, 3006.
[10] H. Yokoi and M. Chikira, *J. Chem. Soc., Dalton Trans.*, 1975, 2101.
[11] H. Kon and N. Kataoka, *Biochemistry*, 1969, **8**, 4757.
[12] W. R. Scheidt, A. C. Brinegar, E. B. Ferro, and J. F. Kirner, *J. Am. Chem. Soc.*, 1977, **99**, 7315.
[13] M. Chikira and H. Kon, *Heterocycles*, 1978, **11**, 253.