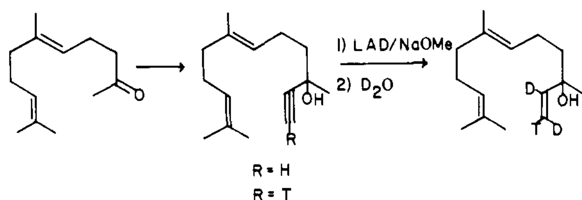
(11) *trans*-Dehydronerolidol was prepared by addition of ethynylmagnesium bromide to *trans*-geranylacetone in methylene chloride. The requisite



[1-^{3}H]dehydronerolidol was then obtained by treatment of dehydronerolidol (0.37 g, 1.68 mmol) in tetrahydrofuran with *n*-butyllithium (2.01 mmol), followed by quenching with 0.20 mL (20 mCi) of [^{3}H]water. The [1-^{3}H]-propargyl alcohol (276 mg, 1.25 mmol) was treated with 158 mg (3.76 mmol) of lithium aluminum deuteride and 406 mg (7.51 mmol) of freshly prepared sodium methoxide in 2.0 mL of tetrahydrofuran at reflux overnight. The reaction was quenched by successive addition of 0.16 mL of D_2O, 0.16 mL of 5% NaOD, and 0.48 mL of D_2O. Ether was added and the products were isolated by filtration through a pad of Celite and evaporation. Purification by PLC (hexane-ether, two developments, 4:1) gave 159 mg of nerolidol, R_f 0.38, 3.58 × 10^7 dpm/mg.
(12) The sequence of deuteride reduction-deuterated water quench was employed in order to ensure essentially 100% deuteration at C-1, as required by the analytical procedure for chiral methyl.[21] The presence of the extra deuterium at C-2 in no way affects the outcome of subsequent reactions.
(13) Based on the (M − H_2O) fragment.
(14) W. W. Epstein and H. C. Rilling, *J. Biol. Chem.*, **245,** 4597 (1970); S. S. Sofer and H. C. Rilling, *J. Lipid Res.*, **10,** 183 (1969); A. A. Kandutsch, H. Paulus, E. Levin, and K. Bloch, *J. Biol. Chem.*, **239,** 2507 (1964); F. Cramer and W. Boehm, *Angew. Chem.*, **71,** 755 (1959); P. W. Holloway and G. Popjak, *Biochem. J.*, **104,** 57 (1967); G. Popjak, J. W. Cornforth, R. H. Cornforth, R. Ryhage, and D. J. Goodman, *J. Biol. Chem.*, **237,** 56 (1962).
(15) the cell-free extract from 1.3 L of *G. fujikuroi* was prepared as previously described[6] except that 25% glycerol (Mallinckrodt, A. R.) was added to improve enzyme stability. The S_{27} fraction was incubated with 1.5 mg (3.17 × 10^7 dpm) of [1,2-$^2H_2$,1-^{3}H]-(1*E*)-nerolidyl pyrophosphate for 4 h at 26 °C.
(16) A portion of the labeled cyclonerodiol was converted to the bis(dinitrobenzoate),[7,17] which was recrystallized to constant activity, indicating an overall 1.1% conversion of nerolidyl pyrophosphate to **1**.
(17) B. E. Cross, R. E. Markwell, and J. C. Stewart, *Tetrahedron*, **27,** 1663 (1971).
(18) E. Wiesenberger, *Mikrochimica Acta*, **33,** 51 (1948).
(19) A control experiment in which unlabeled **1** was subjected to Kuhn-Roth oxidation in the presence of 5 mCi of [^{3}H]water, followed by conversion of the derived acetate to the crystalline *p*-bromophenacyl ester, indicated that oxidation is accompanied by as much as 26% exchange of the methyl hydrogens. Such small amounts of exchange have been observed before[20] and do not present a serious problem. For example, assuming that exchange takes place during oxidation and that the C-1, C-13, C-14, and C-12 (or C-15) methyls contribute eqully to the formation of acetate, that there is no isotope effect for exchange, and that each methyl exchanges only once, 26% exchange would correspond to at most 26% racemization of the chiral acetate. This would change the observed retention of tritium in the fumarase assay by only 6%. Furthermore an isotope effect would tend to protect chirally deuterated and tritiated methyl against exchange, with a consequent reduction in the observed extent of racemization.
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(21) J. W. Cornforth, J. W. Redmond, H. Eggerer, W. Buckel, and C. Gutschow, *Nature (London)*, **221,** 1212 (1969); *Eur. J. Biochem.*, **14,** 1 (1970); J. Luthy, J. Retey, and D. Arigoni, *Nature (London)*, **221,** 1213 (1969).
(22) Determined from the *p*-bromophenacyl ester.
(23) Malate synthase was purified from bakers' yeast by the procedure of Dixon and Kornberg (cf. G. H. Dixon, H. L. Kornberg, and P. Lund, *Biochim. Biophys. Acta*, **41,** 217 (1960); G. H. Dixon and H. L. Kornberg, *Methods Enzmol.*, **5,** 633 (1962)) or using an improved procedure developed by Professor H. Eggerer.
(24) The procedure followed was based on that of Arigoni.[21] Experimental details will be given in the full paper.
(25) J. W. Cornforth, F. P. Ross, and C. Wakselman, *J. Chem. Soc., Perkin Trans. 1*, 429 (1974); J. W. Cornforth and F. P. Ross, *Chem. Commun.*, 1395 (1970); The requisite [1-^{2}H]- and [1-^{3}H]-isopentenals were obtained by pyridinium chlorochromate oxidation[26] of the corresponding alcohols. [1-^{2}H,^{3}H]-(1*R*)-isopentenol was prepared by horse liver alcohol dehydrogenase (HLADH) catalyzed reduction of [1-^{2}H]-isopentenal using 1 equiv of [4-^{3}H]-NADH. The sample of [1-^{2}H,^{3}H]-(1*S*)-isopentenol was obtained from [1-^{3}H]-isopentenol using a catalytic amount of NAD^+ and a 70-fold excess of perdeuterated ethanol in the presence of HLADH.[27]
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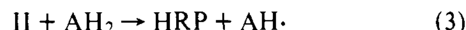
Nuclear Magnetic Resonance Characterization of Compounds I and II of Horseradish Peroxidase

Sir:

Horseradish peroxidase (HRP), which contains iron(III) protoporphyrin, catalyzes the oxidation of wide variety of phenols and aromatic amines by H_2O_2. The widely accepted mechanism depicts[1] the enzymic cycle as

$$\mathrm{HRP} + \mathrm{H_2O_2} \rightarrow \mathrm{I} \quad (1)$$

$$\mathrm{I} + \mathrm{AH_2} \rightarrow \mathrm{II} + \mathrm{AH\cdot} \quad (2)$$


$$\mathrm{II} + \mathrm{AH_2} \rightarrow \mathrm{HRP} + \mathrm{AH\cdot} \quad (3)$$

$$2\mathrm{AH\cdot} \rightarrow \mathrm{A_2H_2} \text{ or } \mathrm{A} + \mathrm{AH_2} \quad (4)$$

This mechanism implies the binding of both H_2O_2 and the electron donor, AH_2, to the enzyme. The nature of the reaction intermediates, compounds I and II, which are respectively two and one oxidizing equivalents above the native enzyme, has been subject of intensive study for many years.[2] I and II are stable enough to have been isolated and characterized by several physical techniques. Magnetic susceptibility,[3] Mössbauer,[4] and resonance Raman[5] studies on II showed it to be in a low-spin ferryl, Fe(IV) state. The Mössbauer spectrum yielded the same iron isomer shifts for both compounds, indicating that I is also in ferryl Fe(IV) state. Electronic absorption spectrum of II is a normal porphyrin type, but that of I resembles the spectrum of a porphyrin π-cation radical.[6] Recently a weak ESR signal at $g = 2.005$ was detected for I to suggest that a free radical is located close to the paramagnetic iron.[7]

In spite of these extensive investigations, the structures of I and II have not been totally resolved. Particularly, it is uncertain whether the additional oxidizing equivalent on I is stored as a radical on the porphyrin ring or on a protein moiety.

We have studied the ^{1}H NMR spectra of these intermediates which allows us to suggest that I and II are, respectively, in high- and low-spin Fe(IV) iron states with different sixth iron ligands and that the free radical may be contained on an amino acid residue close to the heme iron rather than on the porphyrin ring.

In our NMR study, HRP purchased from Toyobo Co. as a liophylized sample ($RZ = 3.4$, isoenzyme c) was used. I (green solution) was generated by adding an equimolar amount of H_2O_2 to 3.0 mM HRP solution in 0.1 M citric acid–0.2 M phosphate buffer at pH 7.0. II (red solution) was prepared from the I solution at pH 9.2[8] in 25 mM borate buffer by the addition of a stoichiometric amount of *p*-cresol. ^{1}H NMR spectra were recorded at various time intervals after the addition of H_2O_2, at various temperatures (5–40 °C) and at different pH's (4–12) with a Varian HR-220/Nicolet TT-100 in a pulsed Fourier transform mode.[9]

^{1}H NMR spectra in the hyperfine shifted region for native ferric HRP, I, and II are compared in Figure 1. The native HRP spectrum, which has previously been reported,[10] is replaced by an entirely different well-resolved spectrum when H_2O_2 was added. The signals at 76.1, 72.1, 59.1, and 50.1 ppm in Figure 1B assigned to the four heme peripheral methyl proton peaks of I decreased in intensity in time with a concomitant increase in intensity of a proton peak at 14.1 ppm, and then the spectrum of the enzyme decayed back to the native enzyme spectrum. Upon immediate addition of *p*-cresol to the I green solution, the spectrum of I disappeared and a rather broad signal at 14.1 ppm grew in, accompanied by reappearance of the native HRP spectrum. Thus we assigned the signal at 14.1 ppm in Figure 1B and 1C to the two of four heme ring methyl proton peaks of II from the integrated intensity. This spectrum of II is also reduced in time, and the

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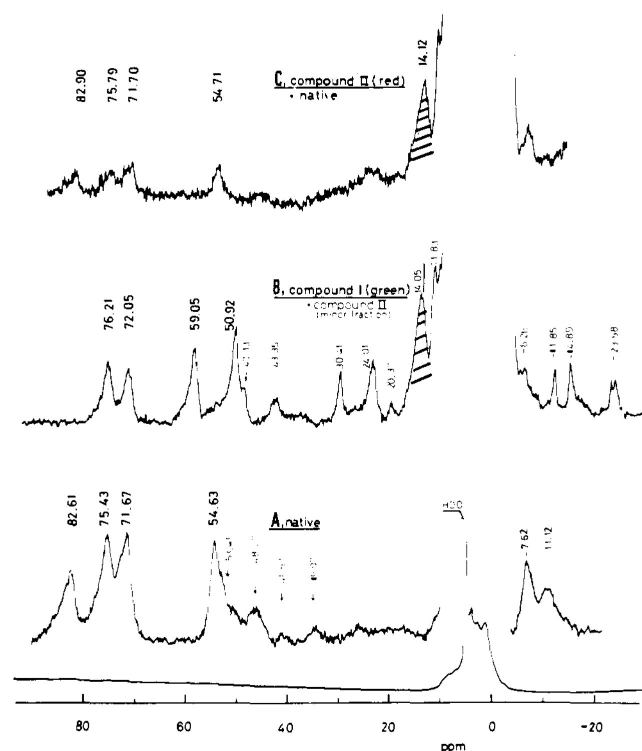

Figure 1. 220-MHz pulse Fourier transform ^1H NMR spectra of native HRP and its reaction intermediates at 20 °C: (A) ferric native HRP at pH 7.0; (B) I at pH 7.0 (the spectrum is contaminated with that of II at 14.1 ppm); (C) II at pH 9.2 (the peaks located at 50-85 ppm are due to recovered ferric native enzyme).

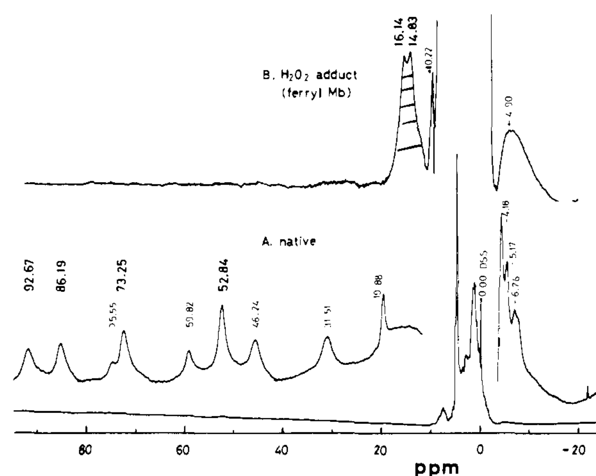

Figure 2. 220-MHz pulse Fourier transform ^1H NMR spectra of horse metmyoglobin and its H_2O_2 adduct at pH 7.9 in 0.2 M phosphate buffer: (A) metmyoglobin; (B) ferrylmyoglobin.

native HRP spectrum was completely recovered within 1 or 2 h. These time progressions of HRP spectra after adding H_2O_2 are consistent with development and decay of I and II (eq 1-3), which was confirmed also by the well-established optical absorption spectra[11] of the solution taken before and after the NMR spectral runs. The line width of each signal for I is rather narrow, compared with that for native HRP.[12] The hyperfine shifted signals in each spectrum of I and II exhibited the temperature dependence of the normal Curie law type, characteristic of single spin state of the heme iron. The pH variation from 4 to 12 caused a slight shift change (3 ppm at most) with pH titration behavior (pK = 5.6) for the spectra of both compounds.[13] We have also observed the 14-16-ppm signals in solution of ferrylmyoglobin (Figure 2), transient species obtained on adding H_2O_2 to metmyoglobin solution.[14] In Figure 1B, the spectrum of I is contaminated with the signal of II at 14.1 ppm, possibly produced by the presence of a small amount of endogenous oxidizable substrate.[15] The signal of II at 14.1 ppm was also generated from I by the addition of other substrates such as indolepropionic acid.[16]

It is to be noted in Figure 1 that the heme peripheral methyl proton peaks exhibit an upfield bias on going from ferric HRP to ferryl I. This trend appears to be in accordance with the NMR observations for ferryl and ferric tetraphenylporphyrins by Felton et al.[17] that the β-pyrrole proton signal at 68.6 ppm for electrochemically generated ferryl high spin (S = 2) TPP chloride progressed in time to 79.4 ppm for ferric high spin (S = $^5/_2$) TPP chloride. It is also worth noting that the well-resolved spectrum of I shows that the source of another oxidizing equivalent in I is not retained on the porphyrin ring as a π cation radical, but rather on the protein moiety located near the heme iron. The π cation radical on the heme ring would produce a much more broadened NMR signal and large shifts far beyond the normal iron-induced paramagnetically shifted spectral region. It has been suggested that the Fe(IV) atom will have a strong dipole-dipole interaction with any nearby radical in I. This dipolar interaction is a source of anisotropic broadening of ESR lines which could account for virtual lack of an ESR spectrum[7] and eventually for facile observation of the rather narrow proton spectrum of I.[12] This iron-radical interaction may be also responsible for the unexpectedly small value of magnetic susceptibility data[3] for the pure ferryliron high-spin (S = 2) state.

The drastic change in the paramagnetic shifts between I and II (or ferrylmyoglobin) may remind one of the similar spectral change of ferric high-spin (S = $^5/_2$, 40-100 ppm) and low-spin (S = $^1/_2$, 10-40 ppm) hemoproteins having different kinds of sixth heme iron ligands.[10] The NMR spectral features of I and II are thus reasonably understood in terms of ferryl high- (S = 2) and low- (S = 1) spin states of the heme iron, respectively. This spin state change may be due to the different heme axial iron ligands in these intermediates. Although the identities of the ligands are not presently established, I and II have been speculatively represented as R·Fe^{IV}=O and RFe^{IV}—OH, respectively, where R· represents an organic free radical on the protein located close to the heme iron, =O an iron bound oxygen atom, and —OH an iron-bound hydroxyl group.[7] The latter structure is based upon the fact that II is formed from I via the addition of both a proton and an electron. The presence of H_2O as the sixth ligand in either of I and II can be ruled out, since the NMR spectra of these compounds did not exhibit any striking pH dependent shift change, as is frequently the case for ferric hemoproteins.[10] The pH titration induced shift with pK = 5.6 is indicative of the presence of a heme linked ionizable group, probably histidine, in the heme environment. The identification of a free radical (R·) in I is now open to further studies.

Acknowledgment. The initial work on the ^1H NMR of ferrylmyoglobin was done with kangaroo myoglobin in collaboration with Dr. M. Ueda, Gifu University, School of Medicine. The authors thank Dr. H. B. Dunford for helpful suggestions on preparation of compounds I and II of horseradish peroxidase. They are also grateful to Drs. T. Yonezawa, T. Iizuka, and Y. Ishimura for their interest and encouragement. This work was supported by grants from Ministry of Education, Japan, and from Toray Science Foundation.

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(8) The addition of a suitable amount of reducing substrate converts compound I to compound II. However, once II is formed, it can compete with I to react with the remaining substrate. Above pH 9, this rate constant is significantly larger for I than II. Therefore, careful work at pH 9.2 enables one to obtain a quantitative yield of II.
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(16) It is therefore likely that the structure of II is independent of the reducing substrate used in its preparation.
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Rearrangement of an Excited β,γ-Unsaturated Ketone Generated by Dioxetane Thermolysis. On the T_1 (π,π^*) and T_2 (n,π^*) Reactivities

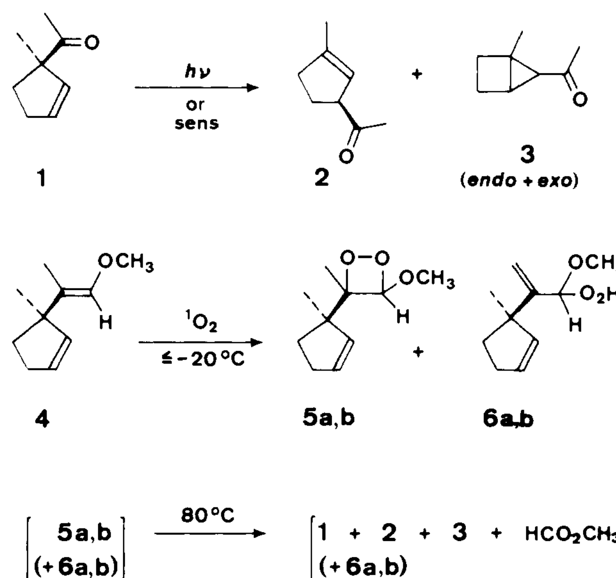
Sir:

Most β,γ-enones undergo two characteristic photoreactions: a triplet sensitized oxadi-π-methane (ODPM) rearrangement which is assumed to occur from the lowest lying $^3\pi,\pi^*$ state, and an allylic 1,3-acyl shift upon direct irradiation which is commonly ascribed to an $^1n,\pi^*$ state.[1-3] However, it has recently been pointed out that all available evidence can also be reconciled with a 1,3 shift from a short-lived T_2 state dominantly n,π^* in character.[2,4-6] As yet, allylic 1,3 shifts from $^3n,\pi^*$ states have only been found with 3-benzoylcyclopentenes which, however, do not undergo the ODPM rearrangement.[7]

We report now on work designed to probe into the reactivity of the $^3n,\pi^*$ state of 3-acetyl-3-methylcyclopentene (**1**), generated by thermal decomposition of the dioxetanes **5a,b**.[8] The product distribution obtained was compared with that from direct and triplet-sensitized photolyses.[9]

The photochemistry of ketone **1**[10] closely parallels that of other 3-acetylcyclopentenes.[2,11] At 25 °C, direct irradiation preferentially yielded the 1,3-acetyl shift product **2**, whereas acetone sensitization favored the ODPM rearrangement to **3** (Table I). As with other β,γ-unsaturated ketones,[4,5] fluorescence of **1** was observed at λ_{max} 410 nm ($\tau_F = 4.5 \pm 0.5$ ns and $\Phi_F = (9 \pm 3) \times 10^{-4}$ in acetonitrile at 25 °C).

Enol ether **4** (0.2 M) was photooxygenated in deuterioacetonitrile at ≤ -20 °C with polymer-bound rose bengal and light from a sodium vapor lamp. Monitoring by NMR[12] indicated a selective attack of $^1O_2$ at the enol ether double bond and formation of the two diastereoisomeric dioxetanes **5a** and **5b**[13] (~12% each) and the hydroperoxides **6a** and **6b** (75%).



When the crude photooxygenation mixture was heated to 80 °C, chemiluminescence identical with the fluorescence of **1** was recorded. The luminescence decreased exponentially with $\tau_{1/2}$ (80 °C) = 375 ± 15 s. After 20 min, when it had reached <10% of its original intensity, ≥90% of **5a,b** had decomposed to methyl formate and the isomers **1**, **2**, and **3** (analysis of the thermolyzed solution by NMR, GLC, and GLC/mass spectrometry; ratio of **1**:**2**:**3**, 95.4:1.9:2.7).[14] The concentration of **6a,b** remained unchanged within a 10% margin in this experiment. The formation of rearranged ketones (**2** and **3**) indicates that dioxetane cleavage had in part produced excited states of ketone **1**, and the chemiluminescence in turn identifies a fraction of these as the excited singlet. Using luminol as a chemiluminescence standard,[15] a fluores-

Table I. Direct and Triplet-Sensitized Photolysis of **1** at 25 °C. Quantum Yields of Conversion and Product Formation[a]

excitation	Φ_{-1}	Φ_2	Φ_3	Φ_2/Φ_3
direct[b]	0.65	0.20	0.04	5.0
sensitized[c]	0.70	0.015	0.46[d]	0.033

[a] Conversions ≤20%. Φ values were measured with argon-degassed solutions in an electronically integrating actinometer: W. Amrein, J. Gloor, and K. Schaffner, *Chimia*, **28**, 185 (1974). Product analysis was by GLC. Overall experimental error was ca. ±7%. [b] 0.1 M in cyclohexane, 313 nm. [c] 0.22 M in acetone, 254 nm. [d] Endo-exo isomer ratio 1:30.

Table II. Rearrangement Products of **1** at 80 °C

starting material	excitation	products[a] 2, %	3, %	ratio of **2**/**3**
1[b]	direct, λ 313 nm, in acetonitrile	15.3	5.6	2.73
1[b]	sensitized, λ 254 nm, in acetone	2.3	74.4	0.031
5a,b[c]	thermal decomposition (20 min) in acetonitrile	(1.9 ± 0.3)	(2.7 ± 0.3)	0.70

[a] Conversions ≤25%. Yields are based on converted starting material. [b] Average values from two runs. [c] Average values from six runs.

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