Comparative Catalytic Activity of Hemin and Hematin in the Breakdown of Methyllinoleate Hydroperoxide and Peroxidation of Methyllinoleate in Methanol

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Received November 18, 1994; accepted February 21, 1995

The breakdown of methyllinoleate hydroperoxide (LOOH) and peroxidation of methyllinoleate (LH) catalyzed by hemin and hematin were studied in 98% methanol. Spectrophotometry was used to follow the breakdown of LOOH. The peroxidation process of LH was monitored by the oxygen consumption. The pK_a for the conversion of hemin to hematin was determined as 7.3 in 98% methanol. The catalytic rates for both processes were found to reach a maximum at pH 8 where about 80% of hemin was in the hematin form. This could be accounted for by the acceleration of the breakdown of LOOH due to binding of hydroxide or methoxide ion to the hemin iron. The reduction in the catalytic activity at a higher pH, where 100% of hemin was in the hematin form, however, suggested that H^+ was also necessary. We propose a new scheme which shows the role of H^+ and OH^- (or CH_3O^-) together with ferryl iron in the catalytic process.

The breakdown of hemin itself was also observed and its catalytic cycle number was estimated as 9. The gradual decomposition of hemin suggests involvement of a Fenton-type mechanism as a minor catalytic process.

Key words lipid peroxidation; hemin; catalytic mechanism; hydroperoxide; pH dependence; ferryl porphyrin

Membrane destruction[1,2] produced by lipid peroxidation causes several diseases.[3-5] Hemin is found to act *in vitro* as a potent lytic agent in several instances[6,7] and its involvement is suggested in *in vivo* lysis process. Heme compounds are ubiquitously distributed in living cells. Forty percent of iron in meat, poultry, and fish is in the form of heme iron.[8] Hemin is known to catalyze the lipid peroxidation and decomposition of lipid hydroperoxides.[9-15] The catalytic activity of hemin changes when the central ferric ion is coordinated by certain ligands such as nitrogenous bases[16] or hydroxyl ion.[17] The effect of OH^- among many ligands seems important and to be a general one since other ferric chelates, such as ferric-histidine,[18] show a similar pH-dependence in terms of its catalytic activity as we found for hemin in this study.The pH effects on hemin activity have been studied by several workers. Kuhn *et al.*[19] found that the optimum pH for the oxidation of oleic acid by hemin was between pH 7 and 8. They also found that hemin precipitation started below pH 6.6. According to Barron and Lyman[16] the linseed oil oxidation by hemin decreased with an increase in pH. Tappel[10] stated that hemoglobin-catalyzed linoleate oxidation increased over 2-fold between pH 7.8 and 9.5. All these works were carried out over a rather narrow pH range and some observations were contradictory. Tappel[9,10] and others[11,12,15] pointed out the importance of breakdown of LOOH[20] for the initiation and acceleration of the peroxidation process. However, there is no report on the pH-dependent breakdown process of LOOH in the presence of hemin. Therefore, it is necessary to study this in detail and also the pH-dependence over a wide range, pH 2—12, with regard to the lipid peroxidation catalyzed by hemin.

The exact definition of hemin and hematin in solution is not very clear: some people used hemin[9,11,13] while others hematin[12,15] in their papers. In the crystalline state, the chloride form is called hemin and the hydroxide form is hematin. However, the axial ligand may change in solution depending on conditions such as pH, concentration, and the ionizing ability of the solvent. In our system only two forms were spectroscopically distinguishable over a wide range of pH, as we will show later. We designate the form at the lower pH as hemin and the one at the higher pH as hematin in this study. It is beyond the scope of this work to determine whether the axial ligand is OH^- or CH_3O^- in "hematin" and whether "hemin" is in a chlorinated or free form. Nevertheless, we use the terms "OH^-" or "hydroxide" for simplicity in the descriptions relating to hematin and let them include the meanings of "CH_3O^-" or "methoxide", respectively, when necessary. In addition to such a definition, the term hemin will also be used to express hemin and/or hematin where the distinction between them is not important.

Usually lipid peroxidation reactions are studied in aqueous suspension or in the micelle state,[11-13,21] but the methanol system also has advantages since it gives a homogeneous solution and more definite spectra compared with the aqueous system.[22]

The present paper reports the results of the peroxidation of LH and the breakdown of LOOH catalyzed by hemin at various pHs in 98% methanol. Possible mechanisms of the catalysis will be discussed based on the results obtained.

Materials and Methods

Materials Methyllinoleate (LH) was purchased from Tokyo Chemical Industry Co., Ltd. This sample is described as fresh LH in the text. LOOH containing methyllinoleate was prepared by two methods. One is autoxidation of the purchased methyllinoleate (LH) by keeping it with a loose cap at room temperature for few weeks. This sample is described as partially oxidized LH, hereafter. The concentration of LOOH was determined from the absorbance at 232 nm (A_{232}) assuming $\varepsilon = 30000\ M^{-1}\ cm^{-1}$.[23] The values are given in the legend to the figures. The other LOOH-containing sample was prepared by mixing fresh LH and purified LOOH, which was separated from the partially oxidized LH by preparative TLC.[24] This sample was used when more quantitative experiments were required.

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Hemin was bought from Tokyo Chemical Industry Co., Ltd. and hematoporphyrin was from Sigma Chemical Company. All other chemicals used were of reagent grade.

Stock Solutions A 0.3 mM stock solution of hemin was prepared by dissolving amorphous hemin in methanol and stirring overnight in the dark. A 1 M fresh LH solution in methanol was kept in a freezer as a stock solution.

pH-Measurements The pH of experimental solutions was measured using a Toa pH-meter (HM-26S) fitted with a Toa, GST 5311S type glass electrode optimized for organic solvents. Sodium hydroxide or perchloric acid was used to change the pH when necessary.

Solvent Throughout the present study we used 98% methanol for the experimental mixture, unless specified or stated otherwise. The presence of 2% water in the experimental mixture was necessary to stabilize the glass electrode of the pH-meter.

Buffer Solution Succinic acid and lithium succinate buffer,[25] suitable for the methanol–water mixed solvent system, was used for the concentration-dependence study of the hematin spectrum under a constant pH. The lithium succinate used in the buffer was prepared by neutralizing 0.01 M succinic acid with 0.01 M lithium hydroxide.

Lipid Peroxidation Assay Peroxidation of methyllinoleate was measured by monitoring oxygen consumption with a Clark type oxygen electrode, Able Model UC-P(G). From the stock solutions of methyllinoleate (1 M) and hemin (0.3 mM), the required volume of each was added to the reaction vessel to give the final concentration as described in the legend to the figures. The reaction was started by injecting hemin into the mythyllinoleate using a microsyringe. The pH of the reaction mixtures was adjusted by the addition of sodium hydroxide or perchloric acid. The total volume of the reaction mixture was 3.5 ml. Fresh solutions were prepared each time and used for the experiments.

The oxygen concentration, just after the start of the reaction, was monitored over time using the oxygen electrode. The peroxidation rates were calculated from the initial decay of the curves.

Spectral Studies Spectra were recorded with a Hewlett Packard Model 8450A diode array spectrophotometer over a wave-length range 200—800 nm. Data were analyzed using a personal computer. The decomposition rates of LOOH and hemin were obtained from the time course measurements of the spectra at various pHs. The spectral change of hemin with pH was also examined to correlate the change in the rates with pH to the hemin status.

Titration of Porphyrins Titration of hemin and hematoporphyrin were performed with the pH-meter as stated earlier in a 50% MeOH–H_2O solvent. Sodium hydroxide solution (9.5 mM) was added into hemin (0.1 mM) or hematoporphyrin (0.1 mM) solution from a burette *via* a pump at a flow rate of 0.5 ml/min. The pH change during titration was monitored on a recording chart.

Results and Discussion

Addition of hemin to the partially oxidized LH led a substantial increase in the oxygen consumption rate compared with fresh LH. A similar result was obtained in more quantitative experiments where fresh LH and purified LOOH were used. As shown in Fig. 1, the initial rate of oxygen consumption increased linearly with [LOOH]. These results suggest that LOOH plays an important role in the hemin catalyzed peroxidation of LH, as pointed out by Tappel.[9,10] Assuming the rate is proportional to [hemin], we obtained an apparent rate constant at [LH] = 0.1 M as follows:

$$k = 100 \times (-d[O_2]/dt)[O_2]_0^{-1}[\text{hemin}]^{-1}[\text{LOOH}]^{-1}$$
$$= 8.09 \times 10^7 \%\ \text{M}^{-2}\,\text{s}^{-1}$$

Where, $[O_2]_0$ is the initial oxygen concentration[26] in the methanol solution saturated under atmospheric conditions.

Figure 2 shows the spectral change in the partially oxidized LH, due to the addition of hemin. The absorption band at 232 nm is for the conjugated diene system of LOOH and the bands at 400 nm (Soret band), 500 and 625 nm (Q-bands) are for hemin absorption. The decrease in the peak intensity at 232 nm with time indicates that hemin causes the breakdown of LOOH. The appearance of the peak at 270 nm can be accounted for by the concomitant formation of an unsaturated carbonyl compound[10,12] similar to 13-keto-9,11-octadecadienoate.[15] The decrease in the peak intensity at 400 nm with time indicates that hemin is decomposed through interaction with LOOH.[10,12,27,28] Hemoglobin,[29] myoglobin[30] and heme[31] were also reported to be destroyed by H_2O_2.

Hemin undergoes rapid decomposition in the presence of partially oxidized LH, but not in its absence (Fig. 3). These results suggest that hemin decomposition is solely due to its interaction with LOOH in partially oxidized LH. Taking the molar extinction coefficient of LOOH and hemin as $3 \times 10^4\ \text{M}^{-1}\,\text{cm}^{-1}$ and $1.5 \times 10^5\ \text{M}^{-1}\,\text{cm}^{-1}$, respectively, we estimated the maximum catalytic cycle number for hemin in methanol to be 9. The catalytic cycle number defined here is the number of LOOH molecules decomposed catalytically by a single hemin molecule before it decomposes. The catalytic cycle number was not changed greatly with the change of [LOOH], as long as the [LH] or [LOOH] was not too high, suggesting that

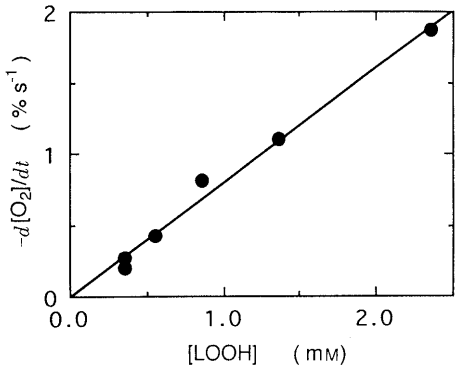

Fig. 1. Peroxidation Rates of Methyllinoleate as a Function of [LOOH] in 98% Methanol

The rates were calculated from the initial slope of the oxygen consumption curves as described in the text. The rates were scaled using the saturated concentration of oxygen under atmospheric conditions. Reaction mixture contained 0.1 M fresh LH, 10 μM hemin and different concentration of LOOH.

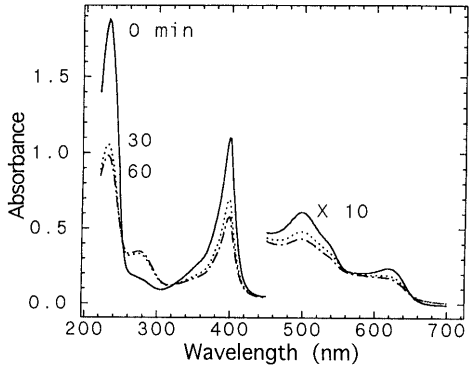

Fig. 2. Time Course Spectra of the Reaction Mixture of Hemin and Partially Oxidized LH in 98% Methanol

Spectra were measured at 0, 30 and 60 min as indicated in the figure, immediately after addition of partially oxidized LH to the hemin solution. Concentrations of partially oxidized LH and hemin in the solution were 0.5 mM and 8 μM, respectively. The initial concentration of LOOH was estimated as 0.06 mM from A_{232}. The reaction mixture in the cuvette was stirred continuously with a magnetic stirrer.

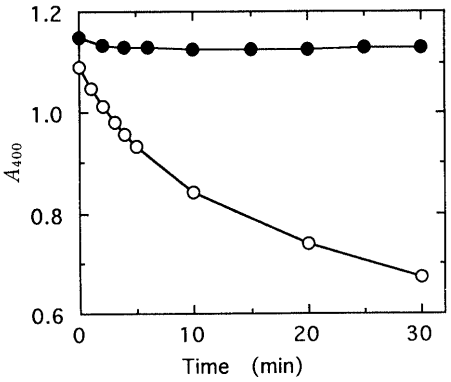

Fig. 3. Decomposition of Hemin in the Presence of Partially Oxidized LH in 98% Methanol

Decomposition of hemin was monitored from the absorbance at 400 nm of the solutions with (○) and without (●) partially oxidized LH at different times. Concentrations of hemin and partially oxidized LH were 8 μM and 0.5 mM, respectively. The initial concentration of LOOH was 0.06 mM.

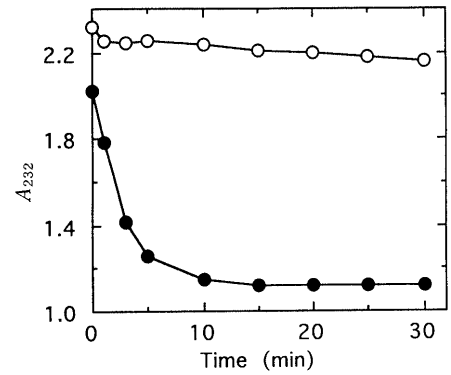

Fig. 4. Effect of pH on the Breakdown of Methyllinoleate Hydroperoxide in the Presence of Hemin in 98% Methanol

A_{232} is the absorbance at 232 nm which reflects the conjugated diene group of LOOH. Concentrations of hemin and partially oxidized LH were 8 μM and 0.5 mM, respectively. The initial concentration of LOOH was 0.06 mM. (○), pH 4.30; (●), pH 8.24. Reactions were started by the addition of partially oxidized LH to the hemin solution.

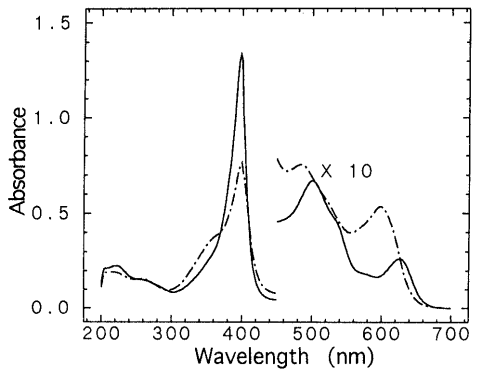

Fig. 5. Change in the Absorption Spectra of Hemin with a pH Change in 98 % Methanol

Concentration of hemin was 8 μM. ——, pH 4.30; –·–·, pH 8.24.

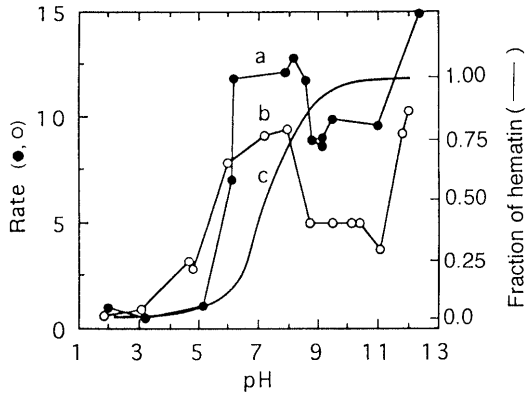

Fig. 6. Spectral Change in Hemin and Its Catalyzing Ability as Functions of pH in 98% Methanol

The breakdown rates (% min^{-1}) of LOOH (a, ●) were calculated from the decay in absorbance at 232 nm. The peroxidation rates (0.1% s^{-1}) of LH (b, ○) were calculated from the oxygen consumption curves as described in the text. The fraction of hematin (c, ——) at various pHs was calculated from the absorbance ratio at 354 to 400 nm. Experimental conditions were as follows. (●), 8 μM hemin + 0.2 mM partially oxidized LH (with LOOH = 0.05 mM); (○), 10 μM hemin + 0.1 M fresh LH (with LOOH = 0.4 mM); (——), 8 μM hemin.

the decomposition of hemin is induced through an intramolecular process involving a hemin–LOOH intermediate or formation of ferrous species, which is very unstable in aerated solution, rather than by the attack of a radical formed at a separate place.

Figure 4 shows the breakdown of LOOH in the presence of hemin at pH 4.30 and 8.24. It is evident that at pH 8.24 the rate is higher than that at pH 4.30. A change in pH also modifies the catalytic species, as shown in Fig. 5. At the lower pH of 4.30 the hemin spectrum shows a sharp Soret band at 400 nm, but at the higher pH of 8.24, a shoulder or bump appears at 354 nm. We checked whether this spectral change was due to simple hydroxylation of hemin or to formation of a μ-oxo dimer. It is true that dimerization or aggregation of hemin takes place in aqueous solution and also in 50% water–methanol solution, since clear broadening of the Soret band was observed at higher concentrations of hemin in these solvents. In 98% methanol solution, however, we could not observe any spectral change over a wide range of concentrations from 10 to 10^{-2} μM at a constant pH adjusted using succinic buffer. It is unlikely that dimers were already formed even at the lowest concentration used, since methanol is a better solvent for most porphyrins than water or 50% methanol. Based on these facts, we assigned the spectrum at alkaline pH to hydroxylated hemin or hematin whereas the spectrum at an acidic pH was assigned to hemin. This assignment is also consistent with the fact that the 354 nm band becomes less distinct at higher concentrations in pure methanol since hemin is an HCl salt which decreases the pH of such an unbuffered solution. The spectra of alkaline hemin in 90% methanol were virtually the same as those in 98% methanol. The spectra in 50% methanol were also not very different from those when the concentration was as low as 0.8 μM. These facts suggest that OH^- is more likely to be the axial ligand in hematin than CH_3O^-.

Hemin was not precipitated at a lower pH in 98% methanol and the spectral changes were reversible with pH change. Moreover, sharp and more definite spectra for hemin and hematin were found in methanol compared with water. Such are the advantages of using methanol as a solvent. Using this advantage of methanol over water, we investigated the pH-dependence of the catalytic activities. The observed results are shown in Fig. 6. Here curve "a" represents the breakdown rates of LOOH

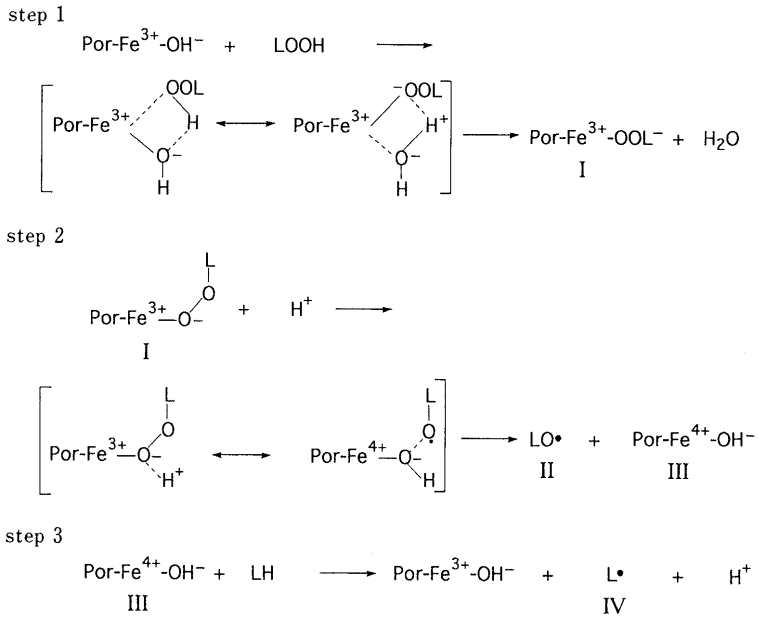

Chart 1

calculated from the initial slope of the decay curves, as shown in Fig. 4. Curve "b" represents the peroxidation rates obtained as described in the materials and methods. Curve "c" represents the fraction of hematin calculated from the ratios of the absorbance at 354 to 400 nm in the hemin spectra at various pHs (Fig. 5). Curve "c" can be fitted to the Hendersen–Hasselbalch equation with pK_a 7.3. The pK_a values for both hemin[32] and heme–enzyme complex[33] in aqueous medium have been reported as 7.6 which is very close to our observations.

The breakdown rate of LOOH (curve "a") is almost constant up to pH 5, when it suddenly increases to reach a maximum at around pH 8. The rate, here, is thirty times higher than that at pH < 5. Comparing curve "a" with curve "c", it is reasonable to deduce that the increase in the rate is due to the formation of hematin. This prompts a new question: why does the rate drop above pH 8.5 while the content of hematin is still increasing? Before we discuss this in detail, we would like to examine the other interesting features that appear in this figure. The behavior of curve "b" is very similar to curve "a" indicating that peroxidation is induced by the breakdown of LOOH. In addition, a sharp increase in the rate at pH 11, seen in both curves, could be attributed to the deprotonation of LOOH or formation of LOO^- which is considered to be more easily oxidized than the parent molecule because of its excess charge.

Now, we return to the question of why the catalytic activity drops (curves "a" and "b") above pH 8.5, before the content of hematin (curve "c") reaches 100%. We considered the following three possibilities.

(a) Dimerization of Hematin Dimerization or aggregation abolishes the catalytic ability of hematin[34] and dimerization is known to occur easily in water.[35,36] However, in our system this was clearly not possible from the concentration-dependence study of the spectrum of hemin in buffered 98% methanol as we stated previously.

(b) Formation of a Dihydroxy Species ($[Por\text{-}Fe(OH)_2]^-$) As the next possibility we checked whether a dihydroxy hematin species is formed with the increase in OH^- concentration, which would be somehow related to the decreasing catalytic activity above pH 8. By means of the potentiometric titration, we found that three moles of sodium hydroxide were required to titrate 1 mol of hemin, whereas 2 mol were required per mole of hematoporphyrin (data not shown). These results strongly suggest that only one hydroxyl ion is bound to the iron of hematin, and, hence, we eliminated the formation of the species $[Por\text{-}Fe(OH)_2]^-$.

(c) Importance of the Simultaneous Existence of OH^- and H^+ The only remaining possibility is the importance of the simultaneous existence of H^+ together with the hydroxylated (OH^-) form of hemin *i.e.*, hematin in the present catalysis system. In 1955 Tappel[10] showed the importance of OH^- bound to hemin, but this did not attract much attention. Instead, recently, Akhtar *et al.*[37] cited that free H^+ is important in the catalytic process. Although these ideas seem mutually contradictory, careful inspection of the individual schemes prompts us to combine them into one scheme as shown below (Chart 1).

In step 1, hematin and LOOH approach each other and finally form an iron porphyrin–peroxide intermediate (I) giving up a molecule of water. A similar intermediate has been suggested for the oxidation processes by hemin,[9,10] catalase, and peroxidase.[37]

In step 2, H^+ attacks the oxygen atom of the intermediate (I), and ultimately I breaks down into LO· (II) and a higher valent ferryl complex (III). This is a modification of the mechanism proposed by Akhtar *et al.* The main difference is which oxygen is attacked by H^+. In the mechanism of Akhtar *et al.* H^+ attacks the oxygen connected to the carbon atom causing an ionic cleavage of the O–O bond to produce LOH. In our mechanism, the oxygen coordinated to the iron atom is attacked by H^+ causing a radical cleavage of the O–O bond. Such a modification is necessary to produce an alkoxyl radical LO· as a product of this step which is considered to be the first major product in the catalytic breakdown of

LOOH by hemin, as claimed by Tappel[10] and Dix *et al.*[15] In spite of such differences in detail, both mechanisms require the involvement of H^+ in the O–O cleavage step. Dawson[39] also discussed the role of H^+ in the mechanism of some peroxidases where distal histidine is regarded as serving as a proton donor.

Step 3 involves hydrogen abstraction and electron transfer steps resulting in the regeneration of hematin with concomitant formation of L· (IV) and H^+. The details of this step are not yet clear, but are acceptable if we take into account the high oxidative ability of ferryl complex (III) and the hydrogen donating nature of the *bis* allylic methylene (–CH=CH–CH_2–CH=CH–) of LH. The possibility of step 3 has also been referred to by Minotti[40] in his review, although he thought more evidence was necessary to establish it beyond doubt.

The radical LO· can initiate the lipid peroxidation chain reaction while the L· radical propagates the reaction in conjunction with oxygen.[9,38]

$$LO\cdot + LH \rightarrow LOH + L\cdot$$

$$L\cdot + O_2 \rightarrow LOO\cdot$$

$$LOO\cdot + LH \rightarrow LOOH + L\cdot$$

The scheme, as shown in Chart 1, indicates that hematin favors the formation of the peroxide intermediate (I) while H^+ favors the formation of LO· (II) by breaking down the intermediate (I). This is why the catalytic activity reaches a maximum at such a nearly neutral region as pH 8 where enough hematin and H^+ can co-exist (Fig. 6).

Although the detection of ferryl iron was not possible in our experiment, the involvement of the ferryl state is widely accepted in ferric-porphyrins such as hematin,[15] cytochrome P-450,[39,41,42] ferric cytochrome c,[43] myoglobin,[30] and metmyoglobin[44,45] during their interaction with H_2O_2 or other hydroperoxides.

Other possibilities such as a Fenton-type redox cycle with hemin have been opposed by many authors.[10,15,46] However, this mechanism may also take place as a minor process because the involvement of the unstable ferrous-porphyrin in the process would explain the accompanying decomposition of hemin (Figs. 2 and 3) or the relatively small catalytic cycle number.

It is interesting to compare the role of OH^- with other iron chelates. In the case of ferric-histidine, we[18] also found that the maximum catalytic activity appeared around the same pH region as observed for hemin.

In summary, we found that hemin reversibly binds an OH^-, with a pK_a of 7.3 in 98% methanol, to produce hematin. In methanol no precipitation of hemin was observed and the spectrum of hemin was sharp and more clearly defined compared with that in water. The rate maximum for the catalytic process around pH 8 indicates that the hydroxylation of hemin together with the simultaneous existence of H^+ is important for the catalytic break down of LOOH.

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20) Abbreviations used: LOOH = lipid hydroperoxide, LH = methyllinoleate, LO· = LOOH derived alkoxyl radical, LOO· = LOOH derived peroxyl radical, L· = LH derived allylic radical, Por-Fe^{3+}-OH = hematin, [Por-Fe^{3+}-$(OH)_2$]$^-$ = hemin dihydroxy anion, Por-Fe^{4+}-OH = ferryl complex or ferryl porphyrin.
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