Singlet Oxygen Generation from the Decomposition of α-Linolenic Acid Hydroperoxide by Cytochrome *c* and Lactoperoxidase†

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ABSTRACT: Generation of singlet oxygen is first investigated in the decomposition of polyunsaturated lipid peroxide, α-linolenic acid hydroperoxide (LAOOH), by heme-proteins such as cytochrome *c* and lactoperoxidase. Chemiluminescence and electron spin resonance methods are used to confirm the singlet oxygen generation and quantify its yield. Decomposition products of LAOOH are characterized by HPLC−ESI-MS, which suggests that singlet oxygen is produced *via* the decomposition of a linear tetraoxide intermediate (Russell's mechanism). Free radicals formed in the decomposition are also identified by the electron spin resonance technique, and the results show that peroxyl, alkyl, and epoxyalkyl radicals are involved. The changes of cytochrome *c* and lactoperoxidase in the reaction are monitored by UV−visible spectroscopy, revealing the action of a monoelectronic and two-electronic oxidation for cytochrome *c* and lactoperoxidase, respectively. These results suggest that cytochrome *c* causes a homolytic reaction of LAOOH, generating alkoxyl radical and then peroxyl radical, which in turn releases singlet oxygen following the Russell mechanism, whereas lactoperoxidase leads to a heterolytic reaction of LAOOH, and the resulting ferryl porphyryl radical of lactoperoxidase abstracts the hydrogen atom from LAOOH to give peroxyl radical and then singlet oxygen. This observation would be important for a better understanding of the damage mechanism of cell membrane or lipoprotein by singlet oxygen and various radicals generated in the peroxidation and decomposition of lipids induced by heme-proteins.

Lipid peroxidation is a well-known example of oxidative damage in cell membranes, lipoproteins, and other lipid-containing structures (*1*). The formed hydroperoxides are easily decomposed by metal ions (e.g., Fe^{2+}, Fe^{3+} Cu^{2+}, Ce^{4+}, etc.), producing alkoxyl and peroxyl radicals, which can further propagate the peroxidation of lipids (*2*−*4*). The whole process is also accompanied by the generation of reactive oxygen species (ROS),[1] such as singlet oxygen ($^1O_2$), superoxide ($O_2^{-\bullet}$), hydroxyl radical ($^\bullet OH$), and hydrogen peroxide, which may be useful or toxic to life (*2*−*4*). The direct emission of $^1O_2$ at 1270 nm has been used as a marker of lipid peroxidation and decomposition (*5*, *6*). However, this use in biological reactions is sometimes problematic because of the low efficiency of $^1O_2$ emission (*7*). Although a detectable amount of ROS can be produced by employing Fe^{2+} and Fe^{3+} ions as the effective triggers of lipid peroxidation and decomposition (*4*, *8*), iron primarily exists in the bound form in heme-proteins in organisms, and the concentration of its free form is rather low (*9*). Therefore, Fe^{2+}- and Fe^{3+}-based research results would inexactly reflect the real situation of biological systems. The use of our recently developed chemiluminescence trap, 4,5-dimethylthio-4′-[2-(9-anthryloxy)ethylthio]tetrathiafulvalene (DAET), may provide the possibility to achieve the sensitive detection of $^1O_2$, since the trap exhibits a highly selective and sensitive chemiluminescence response to $^1O_2$ (*10*).

On the other hand, much effort has been devoted to uncovering the reaction mechanism of heme-proteins with lipid peroxides (*11*−*13*). However, all those studies dealt with only simple or short chain lipid peroxides, instead of long chain polyunsaturated lipid peroxides (the major lipids in the human body) (*11*−*13*). The often used heme-protein is cytochrome *c* (*11*−*12*), which is a globular protein with a heme group coordinately bound to the polypeptide by two thioester linkages and two strong field ligands, a histidine and a methionine residue (*14*). Lactoperoxidase, being capable of catalyzing the oxidation of thiocyanate or halides in the presence of H_2O_2 to release $^1O_2$ (*15*−*18*), is another kind of heme-protein present in organisms, whose heme group is linked to the peptide chain through a disulfide bridge with cysteine residues (*19*). So far, however, the behaviors of these two important heme-proteins have never been investigated in decomposing a long chain lipid peroxide such as α-linolenic acid hydroperoxide (LAOOH). Obviously, conducting such a study would be helpful not only to disclose the physiological function of heme-proteins in lipid peroxidation but also to better understand the damage mechanism of cell membrane or lipoproteins. Herein, we report the results of our research for this purpose.

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[1] Abbreviations: CLA, 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one; Cpd I, compound I; Cpd II, compound II; Cpd III, compound III; DAET, 4,5-dimethylthio-4′-[2-(9- anthryloxy)ethylthio]-tetrathiafulvalene; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; ESI, electron spray ionization; ESR, electron spin resonance; LAH, α-linolenic acid; LAOOH, α-linolenic acid hydroperoxide; $O_2^{-\bullet}$, superoxide; $^\bullet OH$, hydroxyl radical; $^1O_2$, singlet oxygen; RLU, relative light units; ROS, reactive oxygen species; TEMP, 2,2,6,6-tetramethyl-4-piperidone; TEMPO, 2,2,6,6-tetramethyl-4-piperidone-1-oxyl.

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EXPERIMENTAL PROCEDURES

Materials. α-Linolenic acid (LAH) was obtained from Beijing Yanyuan Kema Company. Horse heart cytochrome *c*, bovine lactoperoxidase, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), and 2,2,6,6-tetramethyl-4-piperidone (TEMP) were purchased from Sigma. The concentrations of cytochrome *c* were determined by using a molar absorptivity of 1.06×10^5 M^{-1} cm^{-1} for the heme Soret band maximum at 409 nm (*20*). Lactoperoxidase was assayed spectrophotometrically using a molar absorptivity of 1.14×10^5 M^{-1} cm^{-1} at 412 nm (*21*). The stock solution (200 μM) of chemiluminescence trap DAET was prepared in tetrahydrofuran. 2-Methyl-6-phenyl-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (CLA) (*22*) was purchased from Tokyo Kasei Kogyo Co., Tokyo, and its solution (100 μM) was prepared in water. All other chemicals were local products of analytical grade. Deionized and distilled water was used throughout.

Synthesis of LAOOH from LAH. LAOOH was synthesized by photooxidation using methylene blue as a sensitizer, following a similar procedure described by Di Mascio et al. (*6*). In brief, 1 mL of LAH was mixed with 1 mL of chloroform containing 0.2 mM methylene blue. The mixture was irradiated for 0.5 h under a continuous flux of oxygen at room temperature by an Xe lamp of F-2500 fluorimeter (Hitachi, Japan) with light covering the entire wavelength (the voltage was set to 400 V). Then, the methylene blue was removed and LAOOH separated by silica gel column chromatography by a discontinuous gradient of *n*-hexane: diethyl ether from 5:1 to 1:1 (v/v). LAOOH was characterized by ultraviolet absorption spectra (Techcomp UV-8500 spectrophotometer, Shanghai, China) and ESI-MS (LCMS-2010 Shimadzu, Japan) in the negative ion mode, and its concentration was determined spectrophotometrically using 2.5×10^4 M^{-1} cm^{-1} as the molar absorptivity at 234 nm (*23*).

Electron Spin Resonance (ESR) Detection. TEMP and DMPO were used as the traps for $^1O_2$ and other free radicals (*24*, *25*), respectively. The ESR spectra were recorded with Bruker E-500 for $^1O_2$ and Bruker E-300 for free radicals with the following instrument settings: microwave power 20 mW, modulation frequency 100 kHz, and modulation amplitude 0.1 mT. Typically, a 0.2 mL mixed solution with the final concentrations of 0.05 M $Na_2HPO_4-NaH_2PO_4$ buffer (pH 7.4, referred to the phosphate buffer), 0.4 mM LAOOH, 6.0 μM cytochrome *c* (or 4.0 μM lactoperoxidase), 0.08 M TEMP (or DMPO), and 30% D_2O (v:v) was added into a flat quartz cell, and the ESR spectra were recorded at room temperature. The time delayed before the spectra were recorded was about 2 min. Note that when DMPO was used as the trap for detecting the other free radicals, the system contained no D_2O.

The production of $^1O_2$ from the decomposition of LAOOH by cytochrome *c* or lactoperoxidase was quantified by comparing the ESR integral intensity (calculated by ELEXSYS E500 software) with that from the H_2O_2/NaOCl reaction at pH 7 as a standard (since the yield of $^1O_2$ is near 100% under this condition) (*26*).

Chemiluminescence Detection of $^1O_2$. The generation level of $^1O_2$ from the LAOOH decomposition by cytochrome *c* or lactoperoxidase was also determined by the chemiluminescence method with DAET and CLA as the traps. Briefly, a 1 mL portion of the phosphate buffer containing 30% (v/v) D_2O, 20 μM DAET (or 10 μM CLA) and 0.4 mM LAOOH was placed in a test tube in the chemiluminescence detector. The reaction was initiated by rapid injection of 40 μL of cytochrome *c* (final concentration, 6.0 μM) or 50 μL of lactoperoxidase (final concentration, 4.0 μM), and chemiluminescence was measured as the integrated emission intensity in RLU (relative light units) over the total reaction period (typically 200 s) with a Lumat LB 9507 luminometer (EG & G BERTHOLD, Bad Wildbad, Germany). The yield of $^1O_2$ in the LAOOH system was quantified by comparing the integral of the chemiluminescence intensity with the $^1O_2$ calibration curve constructed according to our previous methods (*10*, *27*). Unless otherwise stated, each of the data was expressed as the mean of three determinations with a relative error of no more than ±10%.

Characterization of Reaction Products of LAOOH with Cytochrome c by HPLC−ESI-MS. After removing the macromolecular species by dialysis, the reaction solution of LAOOH with cytochrome *c* was further separated by HPLC with a column of HiQ sil C18W (4.6 × 200 mm, Jasco, Japan) and a gradient elution (the content of acetonitrile in water was increased linearly from 25% to 40% in the first 5 min, then to 74% during 5 min, and finally to 100% in 5 min), and the reaction products were analyzed by ESI mass spectrometry (LCMS-2010, Shimadzu, Japan).

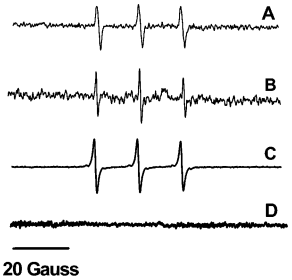


FIGURE 1: ESR signals from different reaction systems containing 0.05 M phosphate buffer (pH 7.4) and 30% (v:v) D_2O. (A) TEMP/LAOOH/cytochrome *c*. (B) TEMP/LAOOH/lactoperoxidase. (C) TEMP/H_2O_2/NaOCl. (D) TEMP/LAOOH (blank). Conditions: 0.4 mM LAOOH, 6.0 μM cytochrome *c*, 4.0 μM lactoperoxidase, 0.08 M TEMP, 0.1 mM H_2O_2, and 0.1 mM NaOCl.

RESULTS AND DISCUSSION

ESR Detection of $^1O_2$ Produced from Decomposition of LAOOH by Cytochrome c or Lactoperoxidase. Cytochrome *c* is known to have the ability of decomposing short chain organic hydroperoxides (e.g., cumene hydroperoxide and *t*-butyl hydroperoxide) giving $^1O_2$ (*11*). In order to better understand the oxidative damage of cell membrane or lipoproteins, this prompts us to examine whether LAOOH (the long chain lipid peroxide commonly present in organisms) could be decomposed generating $^1O_2$. Figure 1A shows the ESR spectrum obtained during the cytochrome *c* induced decomposition of LAOOH in the presence of TEMP. The hyperfine splitting constants of the major signal are $\alpha^N \approx 16.3$ G, $g = 2.0054$, which are identical with those of TEMPO (*24*), indeed indicating the generation of $^1O_2$. Next, we considered whether other heme-proteins like lactoperoxidase possessed a similar decomposing ability. As shown in Figure 1B, the ESR signal characteristic of TEMPO and thus $^1O_2$ production is observed, but the signal intensity is

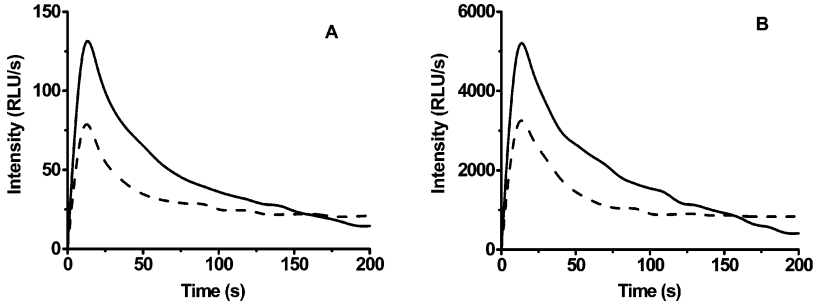


FIGURE 2: Chemiluminescence kinetic curves from different reaction systems containing 0.05 M phosphate buffer (pH 7.4). (A) DAET/cytochrome *c*/LAOOH (solid); DAET/lactoperoxidase/LAOOH (dashed). (B) CLA/cytochrome *c*/LAOOH (solid); CLA/lactoperoxidase/LAOOH (dashed). Conditions: 0.4 mM LAOOH, 6.0 μM cytochrome c, 4.0 μM lactoperoxidase, 20 μM BAET, 10 μM CLA, 200 s of measuring time.

Table 1: Yields of $^1O_2$ in the Reactions of LAOOH with Cytochrome *c* and Lactoperoxidase

system	$^1O_2$ yield by chemiluminescence method (μM)[a]		$^1O_2$ yield by ESR method (μM)[b]
	DAET as trap	CLA as trap	
cytochrome *c*	9.1 ± 0.5	10.8 ± 0.8	7.4
lactoperoxidase	6.3 ± 0.6	7.1 ± 0.5	4.7

[a] The $^1O_2$ concentration in 1 mL of sample solution, 200 s of measuring time; each of the data is expressed as the mean of *n* determinations ± standard deviation ($n = 5$ for cytochrome *c*; $n = 3$ for lactoperoxidase). [b] The $^1O_2$ concentration in 0.2 mL of sample solution, about 2 min of reaction time; the data quoted are the average of two independent experiments. Conditions: 0.4 mM LAOOH, 6.0 μM cytochrome *c*, 4.0 μM lactoperoxidase, 20 μM BAET, 10 μM CLA, 0.08 M TEMP, 0.05 M phosphate buffer (pH 7.4) containing 30% D_2O.

somewhat weaker than that in the case of cytochrome *c* (Figure 1A). However, these results suggest that both of the heme-proteins can decompose LAOOH generating $^1O_2$, which may be attributed to the iron ions in the heme groups. Additionally, the concentrations of $^1O_2$ generated from the LAOOH decomposition by cytochrome *c* and lactoperoxidase were quantified to be 7.4 and 4.7 μM (Table 1), respectively, by comparing the ESR integral intensity with that from the H_2O_2/NaOCl reaction (one of which is depicted in Figure 1C).

Chemiluminescence Detection of $^1O_2$. Due to the high sensitivity and that no excitation light source is required, the chemiluminescence method has become an important means for $^1O_2$ assay (*10*, *27*−*29*). DAET is known to have a strong chemiluminescence response to and high selectivity for $^1O_2$ only, rather than the other ROS. This remarkable property permits $^1O_2$ to be determined accurately in the presence of the other ROS. CLA is also an often used sensitive chemiluminescence trap for $^1O_2$. In this work, the generation of $^1O_2$ from the decomposition of LAOOH by cytochrome *c* or lactoperoxidase was further confirmed by using both DAET and CLA as the chemiluminescent traps.

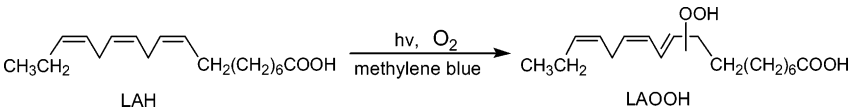
Reactions of LAOOH with cytochrome *c* or lactoperoxidase in the presence of DAET or CLA produced strong chemiluminescence signals, and these signals can be greatly enhanced (about 1.6-fold) by introducing D_2O into the systems, verifying the formation of $^1O_2$ (*30*). Figure 2 shows the chemiluminescence kinetic curves from different reaction systems minus those of the corresponding reagent blanks (DAET/LAOOH and CLA/LAOOH). It can be seen that within the first 200 s the chemiluminescence signal for $^1O_2$ in the presence of cytochrome *c* is higher than that in the case of lactoperoxidase, thus reflecting the more effective decomposition of LAOOH by cytochrome *c* than lactoperoxidase. This may be ascribed to the larger exposure of the heme-group in cytochrome *c* than that in lactoperoxidase (*19*). Furthermore, the reactions of DAET and CLA with $^1O_2$ are very fast (usually completing within a few seconds) (*10*, *27*), and considerable chemiluminescence intensities still remain even after 200 s (Figure 2); therefore the decomposition of LAOOH is a relatively slow reaction.

Similarly, for chemiluminescent quantitative measurements of $^1O_2$, the H_2O_2/NaOCl reaction at pH 7 was used as standard. When DAET was utilized as the trap, a linear equation of $I = 5.66 \times 10^5 [^1O_2] - 0.68$ ($n = 5$, $\gamma = 0.992$) was obtained between the chemiluminescence intensity (I) of DAET and the $^1O_2$ concentration ($[^1O_2]$ in mM) over the range of 2.5×10^{-4} to 0.1 mM. Based on this calibration curve the yields of $^1O_2$ from the decomposition of LAOOH by cytochrome *c* and lactoperoxidase over a period of 200 s were determined to be 9.1 ± 0.5 and 6.3 ± 0.6 μM (Table 1), respectively. With the usage of CLA as the trap, a linear equation of $I = 3.68 \times 10^7 [^1O_2] + 2.59 \times 10^4$ ($n = 5$, $\gamma = 0.998$) was produced over the same $^1O_2$ concentration range, and the yields of $^1O_2$ determined were 10.8 ± 0.8 μM for cytochrome *c* and 7.1 ± 0.5 μM for lactoperoxidase (Table 1), respectively. From Table 1, it can also be seen that the results obtained by both DAET and CLA chemiluminescence methods accord well with each other. The small differences between the chemiluminescence and ESR methods may be attributed to the unidentical measuring time (it is difficult to accurately determine the measuring time in ESR).

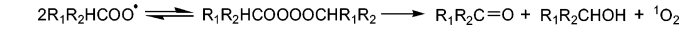
Characterization of LAOOH and Its Decomposition Products by Cytochrome c. In this work LAOOH was prepared by the photooxidation of LAH (Scheme 1). The latter containing three double bonds is a multi-unsaturated fatty acid, whose peroxidation may take place on multiple double bonds. However, our product LAOOH, obtained by column chromatographic separation, displays the 234 nm absorption band indicative of a conjugated diene (Figure S1, Supporting Information), indicating the peroxidation only on a single double bond (*2*, *4*, *31*). Furthermore, the product was also confirmed by ESI-MS analysis ($[M - H]^- = m/z$ 309, and $[M - H_2O - H]^- = m/z$ 291; Figure S2 in Supporting Information). It should be noted that current evidence does not allow the assignment of the peroxidation position.

It has been reported that lipid hydroperoxide decomposition generating $^1O_2$ usually follows the Russell mechanism

Scheme 1: Synthesis of LAOOH from LAH by Photooxidation



Scheme 2: Russell Mechanism for the Self-Reaction of Peroxyl Radicals from Lipid Hydroperoxide Decomposition



(Scheme 2), in which a linear tetraoxide intermediate can be formed and then decomposed to generate an alcohol, ketone (or aldehyde), and molecular oxygen if an α-C hydrogen atom is present (*32*−*34*). To check whether the generation of $^1O_2$ in the LAOOH decomposition by hemeproteins obeys the Russell mechanism, the reaction products of LAOOH with cytochrome *c*, after removing the macromolecular species through dialysis, were examined by HPLC−ESI mass spectrometry. A major ion peak at *m*/*z* 293 was detected (Figure S3, Supporting Information), which corresponds to the expected alcohol molecule ($[M - H]^-$) from LAOOH, whereas the ketone molecule ($[M + H]^+$) at *m*/*z* 293 was found in the positive ion mode (Figure S4, Supporting Information). These data clearly show that the $^1O_2$ generation in the above decomposition also follows the Russell mechanism.

Role of Cytochrome c in LAOOH Decomposition. In order to get insight into the role of cytochrome *c* in decomposing LAOOH, ESR and UV−visible spectroscopy were employed to study the spectral changes of reactants. Figure 3 depicts the ESR spectra from various reaction systems in the presence of the spin trap DMPO under the same conditions. As can be seen, the LAOOH/cytochrome *c* system gave complex signals (Figure 3A). However, by comparing the known values in the Spin Trapping Database at http://EPR.niehs.nih.gov from α-linoleic acid as a reference compound, the major signals may arise from four different spin adducts, whose hyperfine splitting constants (Figure 3A) are as follows: $\alpha^N \approx 16.4$ G, $\alpha^H \approx 23.1$ G for DMPO/LA• (a); $\alpha^N \approx 15.6$ G, $\alpha^H \approx 18.8$ G for DMPO/•LAO (b); and $\alpha^N \approx 14.3$ G, $\alpha^H \approx 10.9$ G for DMPO/LAOO• (c). These results indicate that the radicals, such as LA•, •LAO, and LAOO•, might be involved in the LAOOH/cytochrome *c* system.

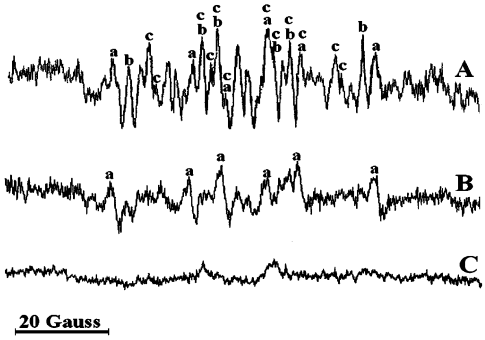


FIGURE 3: ESR spectra from different reaction systems in the presence of DMPO. (A) DMPO/LAOOH/cytochrome *c* (a, DMPO/LA•; b, DMPO/•LAO; c, DMPO/LAOO•). (B) DMPO/LAOOH/lactoperoxidase (a, DMPO/LA•). (C) DMPO/LAOOH (blank). Conditions: 0.4 mM LAOOH, 6.0 μM cytochrome *c*, 4.0 μM lactoperoxidase, 0.08 M DMPO, 0.05 mM phosphate buffer (pH 7.4).

The absorption spectral changes of cytochrome *c* reacting with LAOOH are shown in Figure 4. As can be seen, in the visible region the native ferricytochrome *c* has a maximum Soret band at 409 nm and two weaker absorption bands at 528 and 695 nm (curve 1). The absorption band at 695 nm may be ascribed to the S(Met)−Fe charge transfer transition, which is always used as an indicator that cytochrome *c* is in its native hexacoordinate form (*35*, *36*). Upon reaction with LAOOH, the 409 nm peak decreased greatly with no shift in its position, and after 3 min the decrease changed little with time (curves 2 and 3). It is known that when cytochrome *c* is converted to the ferrous form, the Soret band would slightly red-shift to 416 nm, and prominent α- and *β*-absorption bands would appear at 550 and 521 nm, respectively (*37*). However, such a typical phenomenon (especially the red shift) is not produced in our system, though a rudimentary shape of the α- and *β*-absorption bands can be observed. This implies that after reaction the major existing form of cytochrome *c* might not be in the ferrous form, but possibly in a ferryl species that is responsible for the decrease of the 409 nm peak, which is analogous to the known observations (*11*, *12*). In addition, the reaction of cytochrome *c* with LAOOH results in the disappearance of the 695 nm band, hinting the destruction of the heme hexacoordinate center of cytochrome *c* (*12*, *35*).

Figure 5 depicts the spectral changes of LAOOH in the reaction in the ultraviolet region. It is seen that after reaction the 234 nm absorption peak of LAOOH is decreased, concomitant with the increase of the absorbance around 280 nm, which suggests that some products may possess triene conjugations, perhaps as a result of a keto group conjugated with a 1,3-diene group (*2*). Besides, the spectral change indicates that only a part of LAOOH (about 16%) is decomposed.

Based on the above results, we propose that the decomposition mechanism of LAOOH by cytochrome *c*, which is analogous to the finding of Barr et al. in investigating the decompositions of *t*-butyl hydroperoxide and cumene hydroperoxide,[12] might proceed through the following way (Scheme 3): LAOOH undergoes a monoelectronic reduction

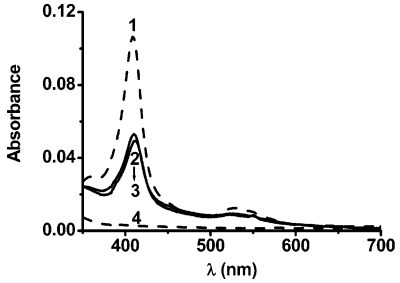


FIGURE 4: The absorption spectral changes of cytochrome *c* in the reaction. (1) Cytochrome *c*. (2 and 3) The reacting mixture of cytochrome *c* and LAOOH at 3 and 40 min, respectively (corrected for the LAOOH contribution). (4) LAOOH. The spectra were recorded in 1 cm quartz cells against the phosphate buffer. Conditions: 1.0 μM cytochrome *c*, 32 μM LAOOH, 0.05 M phosphate (pH 7.4).

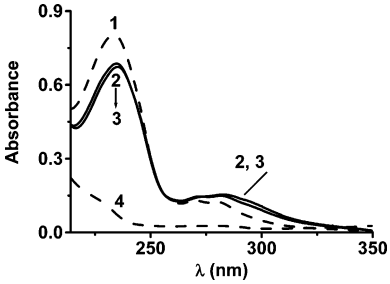
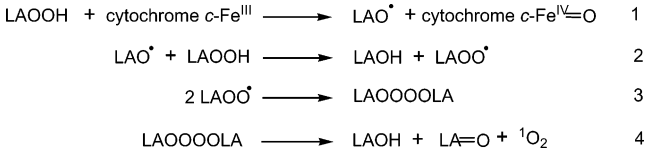


FIGURE 5: The spectral changes of LAOOH reacting with cytochrome *c*. (1) LAOOH. (2 and 3) The reacting mixture of LAOOH and cytochrome *c* at 3 and 40 min, respectively (corrected for the cytochrome *c* contribution). (4) Cytochrome *c*. The other conditions were the same as those in Figure 4.

Scheme 3: Possible Decomposition Mechanism of LAOOH by Cytochrome *c*



by cytochrome *c*, causing the homolytic scission of the O−O bond and thus giving LAO• (reaction 1); LAO• reacts further with LAOOH to generate LAOH and LAOO• through hydrogen abstracting reaction (reaction 2); the formed LAOO• itself couples producing a linear tetraoxide (reaction 3), which decomposes to release LAOH, LA = O, and $^1O_2$ following the Russell mechanism (reaction 4). It should be noted that in our experiment the radical LAO• was not detected by ESR, probably due to its extremely fast hydrogen abstraction (*12*, *38*) and thereby the short lifetime. However, the presence of LAOO• and •LAO detected above by ESR provides the evidence for the involvement of LAO•.

Role of Lactoperoxidase in LAOOH Decomposition. Under the same conditions, the spectral changes from the LAOOH/lactoperoxidase system were also studied by ESR and UV−visible spectroscopy. Compared with the system of DMPO/LAOOH/cytochrome *c*, the ESR spectrum of the DMPO/LAOOH/lactoperoxidase system is much simpler (Figure 3B). In the latter, the only identifiable spin adduct is DMPO/LA•, while the other radicals are not detected, presumably resulting from the low decomposing ability of lactoperoxidase. This accords with the above chemiluminescence result.

The absorption spectrum of native lactoperoxidase, as shown in Figure 6 (curve 1), displays a Soret peak at 412 nm and four weaker absorption bands at 501, 541, 589, and 631 nm, consistent with the previous result (*39*). Furthermore, it is known that in the presence of H_2O_2 the native lactoperoxidase may undergo three consecutive reactions: lactoperoxidase → Cpd I (ferryl porphyrin cation radical) → Cpd II (ferryl without radical) → Cpd III (ferrous−dioxy complex), in which the spectroscopic feature of the unstable intermediate Cpd I is controversial, Cpd II absorbs at about 430 (Soret), 535, and 567 nm, and Cpd III can be characterized by absorptions at about 423 (Soret), 549, and 588 nm (*40*, *41*). In our system, the exposure of lactoperoxidase to LAOOH led to a gradual decrease of the Soret peak at 412 nm in the first 90 min, accompanying first a gradual red-shift to 418 (possible Cpd I, curve 2) and 433 nm (Cpd II, curve 3), then returning to 423 nm (Cpd III, curve 4) and

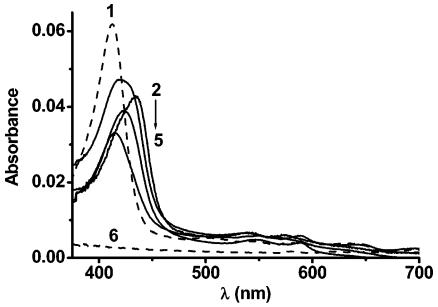


FIGURE 6: The spectral changes of lactoperoxidase reacting with LAOOH. (1) Lactoperoxidase. (2−5) The reacting mixture of lactoperoxidase and LAOOH at 1, 2, 16, and 90 min, respectively (corrected for the LAOOH contribution). (6) LAOOH. The spectra were recorded in 1 cm quartz cells against the phosphate buffer. Conditions: 60 μM LAOOH, 0.5 μM lactoperoxidase, 0.05 M phosphate (pH 7.4).

finally to 413 nm (the native lactoperoxidase, curve 5). After that and up to 24 h no apparent change was detected (data not shown). The intensity decrease of the Soret peak (comparison of curve 1 and curve 5) might arise from the changes in the environment of the heme caused by LAOOH or its decomposition products. A similar quasireversible behavior of Cpd III to the native lactoperoxidase has been mentioned in the literature (*41*).

On the other hand, it is reported that the intermediate Cpd I yielded *via* the two-electronic oxidation of lactoperoxidase by H_2O_2 can be rapidly converted into Cpd II in a monoelectronic way (*42*, *43*). Taken together, we propose that the generation of $^1O_2$ from the decomposition of LAOOH by lactoperoxidase might be initiated by a two-electronic reaction process. Namely, lactoperoxidase reacting with LAOOH produces the ferryl porphyryl radical species (Cpd I) that rapidly abstracts the hydrogen atom from LAOOH to give LAOO•, which then experiences the similar and corresponding reactions in Scheme 3 to produce $^1O_2$.

In summary, the generation of $^1O_2$ from the decomposition of LAOOH by cytochrome *c* or lactoperoxidase is confirmed and quantified by both ESR and chemiluminescence methods for the first time. The decomposition products characterized by ESR and HPLC−ESI mass spectrometry support a Russell mechanism for the $^1O_2$ generation. The changes of cytochrome *c* and lactoperoxidase in the reaction monitored with UV−visible spectroscopy reveal that the decomposition of LAOOH by cytochrome *c* is initiated by a monoelectronic reduction, while that by lactoperoxidase proceeds initiatively by a two-electronic reduction in a heterolytic manner. The results obtained would be helpful for the better understanding of the damage mechanism of cell membrane or lipoprotein by singlet oxygen and various radicals generated in the peroxidation and decomposition of lipids.

SUPPORTING INFORMATION AVAILABLE

UV absorption spectrum and negative ESI mass spectrum of LAOOH; negative and positive ESI mass spectra of the decomposition products of LAOOH. This material is available free of charge via the Internet at http://pubs.acs.org.

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