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Efficacy of Metmyoglobin and Hemin as a Catalyst of Lipid Peroxidation Determined by Using a New Testing System

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A new quantitative approach to investigate the capability of iron heme complexes (HEM), metmyoglobin and hemin, to catalyze lipid peroxidation was elaborated. The oxidation of methyl linoleate in micellar solutions was used as a testing model. The key point was the determination of the rate of free radical generation, R_{IN}, calculated from the rate of oxygen consumption. The HEM catalytic activity was characterized by two independent parameters: by reactivity and by its resistance to degradation. Both parameters were found to be pH-dependent. The reactivity was expressed as the effective rate constant for the reaction of HEM with lipid hydroperoxide. The resistance to degradation was characterized by the rate of the decrease in R_{IN} with time and also by the regeneration coefficient, which shows how many active free radicals can be generated by one molecule of HEM. Both Hemin and metMB were found to be very effective catalysts even at nanomolar concentrations. The effective regeneration of active forms of HEM was observed. The catalytic activity of HEM was rapidly reduced with time. The kinetic scheme of the process under consideration was suggested, and this was applied for kinetic computer simulations.

KEYWORDS: Myoglobin; hemin; metmyoglobin; lipid peroxidation; free radicals; kinetics

INTRODUCTION

The great interest in the studies of catalytic activity of Fe heme complexes (HEM) is caused by several reasons. Natural HEM, hemoglobin (HB) and myoglobin (MB), acting as an oxygen carrier under normal physiological conditions, can be toxic at some pathologies (*1*−*3*). The toxicity of HEM is commonly explained by their ability to generate active free radicals, which results in the degradation of biologically significant substances such as DNA, proteins, and lipids (*4*−*7*). The catalytic activity of HEM, first of all of MB and HB, is considered in connection with the problem of spoiling of muscle foods (meat, fish, etc.) during their storage (*8*−*10*). Also, the role of MB in undesirable oxidative processes in the stomach upon consumption of fatty meat food has been considered (*4*, *5*, *11*−*13*). Among the processes induced by HEM, the chain oxidation of highly reactive polyunsaturated fatty acids (PUFA) has attracted the most considerable interest. In particular, the chain peroxidation of PUFA induced by MB and HB is believed to be the main reason for the spoiling of meat and other muscle foods (*8*−*10*).

Despite a great number of published works concerning lipid peroxidation induced by HEM (see refs *1*, *7*, and *14* for reviews), many aspects of the problem under consideration remain unclear. Much attention has been paid to elucidation of specific forms of HEM responsible for HEM catalytic activity (*1*, *4*, *5*, *7*, *11*, *15*−*17*). It was found that HEM react readily with peroxides (*4*, *11*, *17*−*20*). The very significant observation that should be mentioned is that HEM display the catalytic activity only in systems containing at least traces of peroxides, H_2O_2 (*1*, *7*, *21*) or lipid hydroperoxides (LOOH) (*1*, *6*, *21*−*23*). It has been repeatedly reported that the efficacy of MB as a catalyst of lipid peroxidation is significantly higher at acidic pH, that is, under conditions typical of stomach and meat products (*1*, *5*, *11*, *17*). Meanwhile, the majority of the works devoted to the kinetics of lipid peroxidation induced by HEM are of qualitative character and thus not too informative. In particular, information on the rate of the generation of active free radicals initiating lipid peroxidation and about the change in catalytic activity of HEM in this process with time has never been reported. No general approach to these problems has been suggested yet.

The current work is devoted to elaborating such a quantitative approach based on the theory of chain free radical oxidation. The chain oxidation of methyl linoleate (ML) in micellar solution was suggested as a testing model. The kinetics of oxidation are followed by steady monitoring of oxygen consumption. Along with other things, the model allows determining the rate of free radical generation at any moment of time as well as following the degradation of HEM catalytic activity with time. The quantitative parameters characterizing the activity of HEM as a catalyst of lipid peroxidation have been suggested.

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Scheme 1 General Kinetic Scheme of the Chain Free Radical Oxidation of LH

(0)	Initiation $\longrightarrow$ r$^\bullet$	(R_{IN})
	r$^\bullet$ + O_2 $\longrightarrow$ r$O_2^\bullet$	
	r$O_2^\bullet$ + LH $\longrightarrow$ rOOH + L$^\bullet$	
	L$^\bullet$ + O_2 $\longrightarrow$ L$O_2^\bullet$	
(1)	L$O_2^\bullet$ + LH $\longrightarrow$ LOOH + L$^\bullet$	(k_1)
(2)	L$O_2^\bullet$ + L$O_2^\bullet$ $\longrightarrow$ products	($2k_2$)

These studies were undertaken for metmyoglobin (metMB), the oxidized form of MB, and its protein-free analogue, Hemin.

MATERIALS AND METHODS

Horse heart metmyoglobin (metMB), methyl linoleate, and Triton X-100 were purchased from Sigma. Hemin was obtained from Carl Roth, Karlsruhe, Germany. The water-soluble initiator 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Polysciences. Sodium phosphates of highest quality, NaH_2PO_4 and Na_2HPO_4, used to prepare buffer solutions were purchased from Merck. The buffer solutions with pH within the range of 4.8−8.0 were prepared by mixing 50 mM solutions of NaH_2PO_4 and Na_2HPO_4 without adding any acid or base. In turn, the solutions of the individual phosphates were prepared with doubly distilled water and were purged from traces of transition metals by Chelex-100 resin (Bio-Rad). The standard testing system was composed of 50 mM buffer, 50 mM Triton X-100, and 2−20 mM ML. Hemin and metMB were added as stock solutions. Hemin was dissolved in aqueous DMSO, and metMB was dissolved in 50 mM phosphate buffer, pH 7.4. The concentration of metMB in stock solution was controlled by its optical density at 410 nm (Soret band). The extinction coefficient ϵ_{410} was found to be as much as 1.70×10^5 M cm^{-1}, in good agreement with that reported in the literature [1.75×10^5 M cm^{-1} (*7*) and 1.5×10^5 M cm^{-1} (*4*)].

The kinetics of oxygen consumption accompanying ML oxidation were studied with a computerized 5300 Biological Oxygen Monitor (Yellow Springs Instruments Co.) with a Clark electrode as a sensor. The rate of oxidation was measured as a slope of [O_2] trace. In more detail, the protocol of the determination of the kinetics of oxygen consumption has been described elsewhere (*24*, *25*). Experiments were conducted at 37.0 ± 0.1 °C and at pH 7.40 ± 0.02, unless otherwise indicated. ML was added to a preliminarily thermostated micellar solution of Triton X-100 in buffer. Monitoring was started in 3−5 min after ML addition. Hemin or metMB was added to a work cell as a stock solution by using a Hamilton microsyringe under condition of steady monitoring.

The preoxidized ML containing a certain amount of ML hydroperoxide (LOOH) was prepared by autoxidation (without adding an initiator) of homogeneous ML at 60 °C in a work cell of a sensitive microvolumometer (see ref *26* for experimental details). Under these conditions, LOOH is the only source of active free radicals and the rate of oxidation increases linearly with time. The concentration of LOOH accumulated could be easily calculated from the rate of oxidation as this has been described in our previous work (*27*). The kinetic computer simulations were performed by using the Kinetics program (*24*).

RESULTS AND DISCUSSION

Testing Model. The oxidation of ML in Triton X-100 micellar solution occurs as a developed chain free radical process similar to the oxidation PUFA in homogeneous solutions (*28*−*30*) and in micro-heterogeneous systems (*25*, *31*, *32*). This may be described by the classical kinetic **Scheme 1**. At moderate temperatures (≤60 °C), LOOH was found to be actually the only molecular product of linoleate oxidation (*33*, *34*). This has also been recently demonstrated during the oxidation of linoleic acid induced by MB (*11*). Under these conditions, the rate of ML oxidation, R_{OX}, can be characterized both by the rate of LOOH accumulation and by the rate of oxygen consumption. As it follows from **Scheme 1**, the rate of chain oxidation is determined by eq 1

$$R_{OX} = \frac{k_1}{\sqrt{2k_2}}[\mathrm{LH}]\sqrt{R_{IN}} \quad (1)$$

irrespective of the initiation mechanism. The rate of free radical generation (initiation), R_{IN}, may be determined by the inhibitor method from the induction period (t_{IND}) of oxidation in the presence of a known concentration of a standard antioxidant (*36*), for instance, 6-hydroxy-2,2,5,7,8-pentamethylbenzochroman (HPMC).

$$R_{IN} = 2[\mathrm{HPMC}]/t_{IND} \quad (2)$$

Unfortunately, this method of determining R_{IN} is not suitable for ML oxidation induced by HEM as in this case, as it will be shown below, R_{IN} rather rapidly changes with time. The validity of eq 1 for our testing system was verified in a special series of experiments with AAPH as an initiator. In accordance with eq 1, R_{OX} is proportional to ML concentration and the square root of R_{IN} (not shown). The parameter $k_1/(2k_2)^{0.5}$ required for further determinations was calculated from R_{OX} by using eq 1. Protocols of the related procedures have been repeatedly reported in the literature (see, for example, ref *28*). For our system, the value of $k_1/(2k_2)^{0.5}$ was found to be 0.47 (Ms)$^{-0.5}$.

Oxidation of Nonoxidized ML. Both metMB and Hemin taken in rather low concentrations effectively induce ML oxidation as exemplified by **Figure 1**. It may be seen that the concentration of oxygen consumed and hence the number of molecules of ML oxidized exceeded the concentration of Hem added by at least 2 orders of magnitude. Thus, in the run presented in **Figure 1A**, adding 100 nM Hemin causes the oxidation of 120 μM ML, that is, 1200 molecules of ML per molecule of Hem. In principle, two factors causing such an effect should be considered: (1) Because ML oxidation is a chain process, the formation of one active free radical can result in the oxidation of a significant number of substrate molecules. (2) In the process under consideration, HEM acts as a catalyst, that is, one molecule of HEM is capable of producing more than one active free radical. The latter is possible owing to effective regeneration of a catalytically effective form of HEM. As will be shown below, both factors are significant and can be separated quantitatively.

Unambiguous evidence that the intensive oxygen uptake in the system under study is just due to the chain oxidation of ML was obtained in the experiments with the addition of HPMC, which has a potent antioxidative action caused by its high reactivity to chain-carrying peroxy radicals (*35*). The example of such an experiment is given in **Figure 2**. It is seen that R_{OX} decreased sharply as soon as HPMC was added. Later, as the antioxidant was consumed in reactions with the peroxy radical, R_{OX} increased progressively with time; by the end of the induction period the rate reached nearly the same high value that had existed before antioxidant addition.

Two regions may be distinguished in the kinetic curves of oxygen consumption (**Figure 1**). At the starting stage, R_{OX} increased with time. When R_{OX} peaked, it started to decrease with time, in the case of Hemin, up to a very low value of ca. 10 nM s^{-1} that is typical of the process in the absence of HEM (**Figure 1A**). In the case of metMB, the reduction of R_{OX}

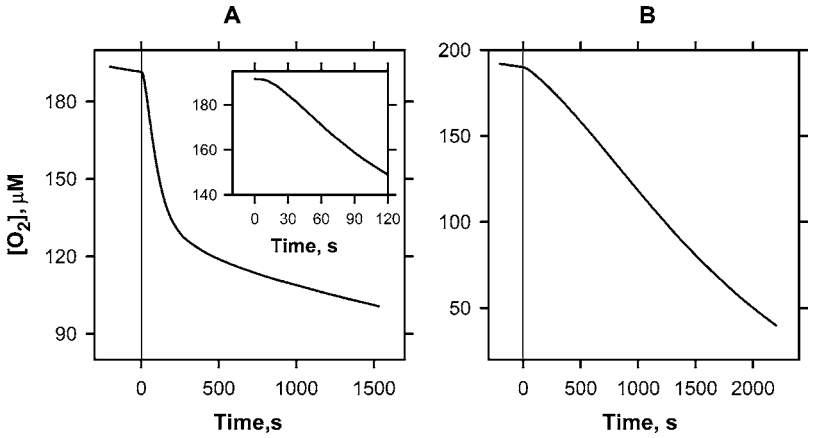

Figure 1. Kinetics of oxygen consumption during the oxidation of 5 mM ML in 50 mM micellar solution of Triton X-100, pH 7.40, induced by 50 nM Hemin (**A**) (inset shows the starting part of the kinetic curve) and induced by 1 μM metMB (**B**). HEM was added at $t = 0$.

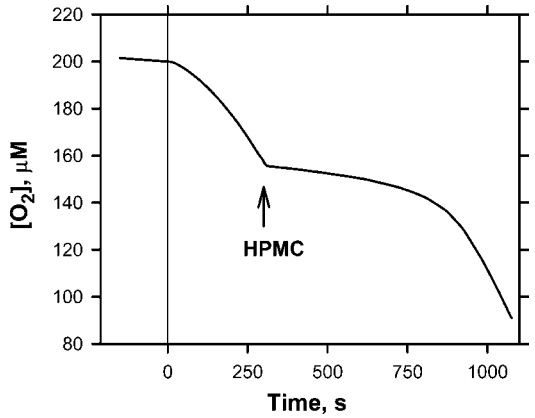

Figure 2. Effect of addition of 2 μM HPMC on the oxidation of 8 mM ML in 50 mM micellar solution of Triton X-100, pH 7.40, induced by 1 μM metMB. MetMB was added at $t = 0$.

occurred much more slowly, and by the moment when dissolved oxygen was almost exhausted, R_{OX} remained rather high (**Figure 1B**). A further decrease in R_{OX} was observed after temporary interruption of monitoring and a partial restoration of oxygen (not shown). The maximum achievable value of R_{OX} increased when the concentrations of both HEM and ML increased (**Figure 3**).

Let us next consider the reasons for the increase in R_{OX} with time during the starting stage of the process and its consequent reduction. As mentioned in the Introduction, HEM display catalytic activity only in the presence of peroxides, in particular, of lipid hydroperoxide, LOOH. The crucial role of LOOH as a factor determining R_{OX} follows also from our experiments. The rate of oxidation measured after the repeated addition of Hemin (after the almost complete loss of its catalytic activity during the first run) was significantly higher than that determined after its first addition (**Figure 4**). This effect is evidently due to the elevated concentration of LOOH accumulated during the first run. As will be shown below, the rate of oxidation of preoxidized ML increases when the concentration of LOOH increases. The addition of triphenylphosphine, a well-known destroyer of hydroperoxides (*36*), results in almost complete inhibition of ML oxidation induced by HEM (not shown). We may conclude that the increase in R_{OX} with time during the starting stage of the process occurs thanks to the growth of LOOH concentration.

As for the decrease in R_{OX} with time, this is evidently caused by the reduction of the concentration of one of the reagents, ML, O_2, or HEM. During a single kinetic run, nearly 100 μM O_2 is commonly consumed (**Figure 1**), which means that nearly 100 μM ML is oxidized. As the starting concentration of ML varies within the range from 2 to 20 mM, not more than 5−0.5% ML is consumed during a run. This evidently cannot significantly change R_{OX}. It is somewhat more realistic to consider the reduction of $[O_2]$ in the course of a kinetic run as a reason for the decrease in R_{OX} with time. To test the validity of this idea, the influence of starting oxygen concentration on R_{OX} was studied (the starting value of $[O_2]$ was altered by short-term expulsion with argon or pure oxygen). It was found that the decrease in $[O_2]$ from 360 to 100 μM resulted in only a moderate decrease in R_{OX}. For example, when $[O_2]$ decreased from 360 to 100 μM, the maximum value of R_{OX} during the oxidation of 5 mM ML induced by 50 nM Hemin changed from 580 to 470 nM s^{-1}, that is, by ca. 20% only. These data suggest that the decrease in the catalytically active form of HEM is the main reason for the decrease in R_{OX} with time.

The efficacy of HEM as an inducer (catalyst) of ML oxidation is governed by two independent factors: (1) the rate of free radical generation by HEM (catalytic activity) and (2) the duration of the catalytic process that is determined basically by the capability of a catalytically active form for regeneration. Although the above factors can be qualitatively estimated by using experiments with "fresh", nonoxidized ML, these are not suitable for quantitative determinations. The main problem is the progressive increase in [LOOH] in the course of a kinetic run; the latter makes the kinetic analysis rather complicated. As will be shown in the next section, using preoxidized ML containing a known, high enough concentration of LOOH is much more promising.

Oxidation of Preoxidized ML. The main advantage of using preoxidized ML for quantitative determinations is that the change in LOOH concentration occurring in the course of a kinetic run is negligibly small, especially at the starting stage of the process. This simplifies the kinetic analysis and makes the determination of parameters characterizing the catalytic activity of HEM more reliable. In contrast to the oxidation of nonoxidized ML, R_{OX} peaks almost immediately after HEM addition. For instance, in the experiment with 250 nM metMB and preoxidized ML containing 0.25 mM LOOH, R_{OX} peaked at ca. 20 s. By this moment only 10 μM LOOH was additionally accumulated, which is only 4% of the starting concentration.

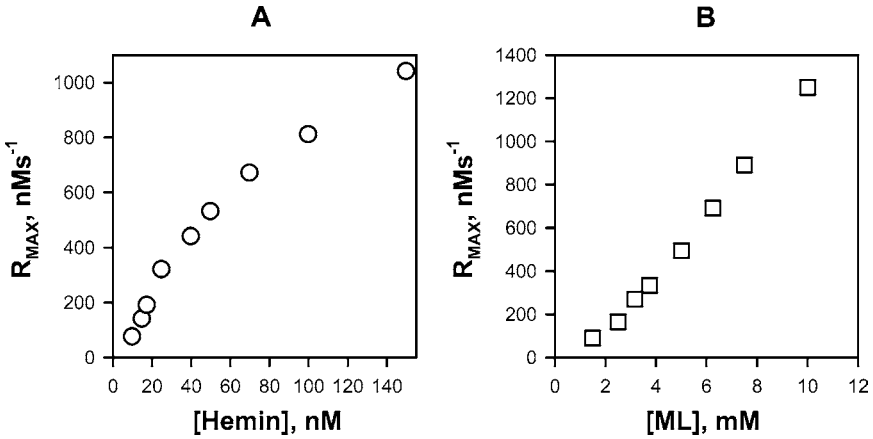

Figure 3. Dependence of the maximal rate of oxygen consumption, R_{MAX}, on reagent concentrations during the oxidation of ML in 50 mM micellar solution of Triton X-100, pH 7.40, induced by Hemin: (**A**) R_{MAX} against [Hemin] at [ML] = 5 mM; (**B**) R_{MAX} against [ML] at [Hemin] = 50 nM.

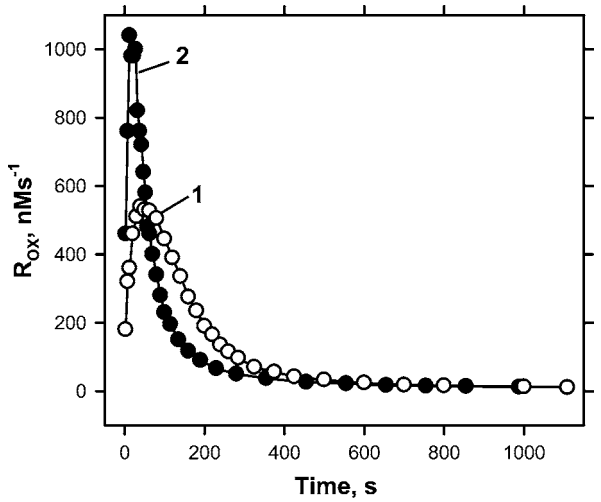

Figure 4. Effect of repeated addition of 50 nM Hemin on the oxidation of 5 mM ML in 50 mM micellar solution of Triton X-100: 1, first addition; 2, second addition.

The rate of free radical generation (rate of initiation), R_{IN}, can be calculated from R_{OX} at any moment of time by using eq 3 deduced directly from eq 1.

$$R_{IN} = \left(\frac{R_{OX}}{(k_1/\sqrt{2k_2}[LH])}\right)^2 \quad (3)$$

On the assumption that the initiation occurs by the interaction of HEM with LOOH (*4*−*6*, *21*, *22*)

$$Hem + LOOH \rightarrow LO_2^{\bullet} + \text{products}\ (k_{IN})$$

$R_{JN} = k_{JN}$ [LOOH] [Hem], and the rate constant k_{IN} for initiation may be calculated from the starting value of R_{IN}

$$k_{IN} = R_{IN}/[Hem] \times [LOOH] \quad (4)$$

A value of k_{IN} determined in such a way is practically independent of the concentration of reagents, which vary in the case of Hemin in the range between 2 and 20 mM (ML), between 20 and 100 nM (Hemin), and between 0.1 and 0.5 mM (LOOH). Thus, k_{IN} may be considered as an appropriate and reproducible characteristic of HEM catalytic activity. The values of k_{IN} are listed in **Table 1**. It should be noted that at pH 7.4 k_{IN} for Hemin exceeds that for metMB by nearly 2 orders of magnitude.

Table 1. Kinetic Parameters Characterizing the Catalytic Activity of Hemin and MetMB during the Oxidation of ML in Micellar Solution of Triton 100-X at 37 °C

HEM	pH	k_{IN}, M^{-1} s^{-1}	regeneration coeff, *f*	k_D, s^{-1} [a]
Hemin	7.4	10900 ± 1100	90–150	0.014 ± 0.002
	4.8	7200 ± 900	50–80	0.039 ± 0.005
metMB	7.4	135 ± 15	>12[b]	0.0014 ± 0.0002
	4.8	1990 ± 120	40–70	0.022 ± 0.003

[a] Related to the starting part of kinetic curves. [b] Significant oxygen consumption was detected by the moment when oxygen in the reaction mixture was almost exhausted.

Two approaches may be suggested to characterize the resistance of HEM to the loss of catalytic activity with time (degradation). Both are based on the kinetics of R_{OX} reduction. As has been above suggested, the decrease of the concentration of an active form of HEM is likely the only reason for the decrease in R_{OX} with time. The first approach is based directly on the decrease in R_{IN} in the course of a kinetic run. (We note that R_{IN} may be calculated at any moment of time from R_{OX} by using eq 3.) As is shown in **Figure 5**, the starting stage of reducing R_{IN} with time is well described by the first power equation (ln R_{IN} is directly with time), but at later time, the rate of R_{IN} reduction decreases and this linear law does not work. It should be noted that the slope of the plots in **Figure 5**, $tg\alpha$, practically does not change with varying HEM concentration. The monomolecular rate constant $k_D = tg\alpha$ may be considered as an effective parameter characterizing the tendency of HEM to degradation. The values of k_D are listed in **Table 1**.

A more general approach to the estimation of HEM resistance is based on the graphic calculation of the integral

$$n = \int_0^{\infty} R_{IN}\, dt \quad (5)$$

The integral is just the square under the plot of R_{IN} versus time. The value of *n* in eq 5 denotes the total number of active free radicals (in concentration units) that have been generated throughout the kinetic run. For practical use, *n* value should be referred to the starting concentration of HEM

$$f = n/[HEM] \quad (6)$$

The *f* value may be considered as a coefficient of HEM regeneration (a number of catalytic cycles); it shows how many times on average one molecule of HEM participates in the

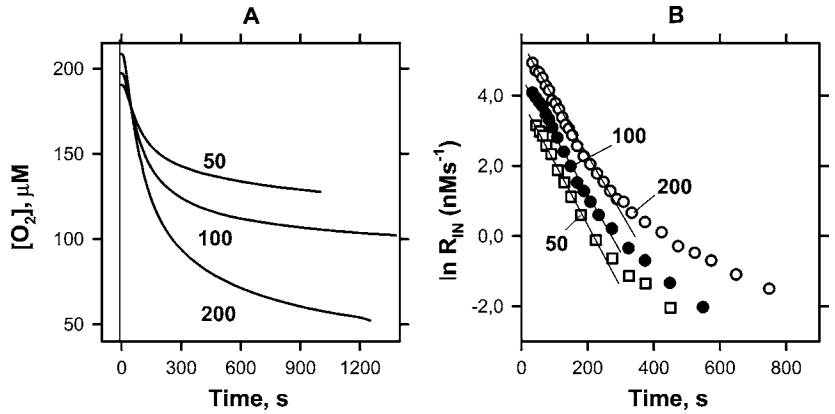

Figure 5. Oxidation of 5 mM preoxidized ML ([LOOH] = 0.25 mM) induced by various concentrations of metMB (indicated at plots in nM) in 50 mM micellar solution of Triton X-100, pH 4.80: (**A**) original kinetic curves (metMB was added at $t = 0$); (**B**) plots of ln R_{IN} against time (R_{IN} was calculated from original kinetic curves by using eq 3).

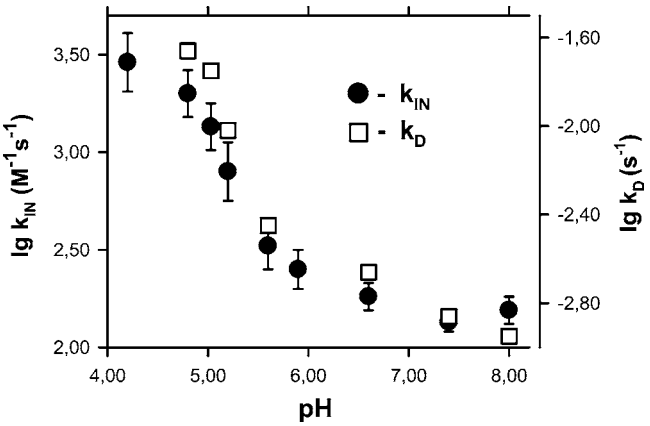

Figure 6. Effect of pH on k_{IN} and k_D determined during the oxidation of ML induced by metMB in 50 mM micellar solution of Triton X-100.

generation of active free radicals. The *f* values calculated are presented in **Table 1**. It may be seen that in all the cases *f* exceeds significantly 1; sometimes *f* ranges up to 100 or more. Thus, HEM may be considered as a genuine catalyst of the lipid peroxidation. Contrary to k_{IN}, the value of *f* calculated somewhat changes when reagent concentrations vary. In more detail, this problem will be considered in the next section. It should be noted that the method for the calculation of *n* and *f* based on the integration is universal; it may be also applied both for preoxidized and "fresh" ML.

The earlier presented data were related to pH 7.4. It follows from **Table 1** and **Figure 6** that the catalytic activity of metMB expressed as k_{IN} increases significantly at acidic pH. This is in line with the data reported in the literature (*4*, *5*, *17*). In contrast to metMB, the catalytic activity of Hemin remains almost unchanged (**Table 1**). At the same time, the tendency of both Hemin and metMB to degradation expressed as k_D increases at acidic pH. The effect is less pronounced for Hemin (**Table 1**).

Several hypothetic versions of the mechanism describing lipid peroxidation catalyzed by HEM have been suggested in the literature (*7*, *14*, *15*). The key point of most of them is the involvement of lipid hydroperoxides. To the first approximation, the process of catalytic lipid oxidation may be presented by **Scheme 2**, which is actually a special version of the more general **Scheme 1**. HemI and HemII in **Scheme 2** are two forms of HEM, reduced and oxidized; their structures are not specified. Although **Scheme 2** is somewhat oversimplified as compared to more realistic "chemical" schemes suggested in the literature, it reflects the most essential kinetic features of the process under

Scheme 2. Lipid Peroxidation Catalyzed by HEM

(3)	$HemI + LOOH \longrightarrow LO_2^{\bullet} + HemII$	(k_3)
(3a)	$HemII + LOOH \longrightarrow LO_2^{\bullet} + HemI$	(k_{3A})
(1)	$LO_2^{\bullet} + LH \longrightarrow LOOH + L^{\bullet}$	(k_1)
(5)	$L^{\bullet} + O_2 \longrightarrow LO_2^{\bullet}$	(k_5)
(2)	$LO_2^{\bullet} + LO_2^{\bullet} \longrightarrow$ products	(k_2)
(4)	$HemII \longrightarrow$ catalytically non-active products	(k_4)

consideration and is quite suitable for kinetic simulations. Among other things, the application of **Scheme 2** allows one to avoid deciding between hypothetical variants of more "chemical" schemes. The initiation occurs thanks to the multiple repetitions of reactions 3 and 3A accompanied by the transformation of two forms of HEM, HemI ↔ HemII. Although the experimentally determined k_{IN} is an impartial characteristic of HEM catalytic activity, k_{IN} cannot be attributed to a certain elementary reaction; this may be considered as an effective value only. As is evident from **Table 1**, the number of cycles of interconversion HemI ↔ HemII is much more than one. It is believed that both forms of HEM, reduced and oxidized, participate in the generation of active free radicals really from the very beginning of the process. The effective rate constant k_{IN} is evidently a combination of k_3 and k_{3A}, and it also depends on the current concentrations of HemI and HemII. This problem will be considered in more detail below.

To the first approximation, k_4 in **Scheme 2** may by associated with the experimentally determined value k_D; however, the nature of reaction 4 remains unclear. Two pathways of metMB degradation have been suggested in the literature: transformation into catalytically inactive hemichrome (*7*, *14*) and Hem moiety destruction accompanied by free iron release (*7*, *14*, *15*). In the current work, the mechanisms of these processes have not yet been elucidated in detail. Nonetheless, our data allow us to exclude some hypothetic versions from consideration. First of all, it should be noted that HEM degradation occurs in the presence of an oxidizing lipid, whereas in the absence of ML stock solutions of both metMB and Hemin remain unchanged upon storage at 4 °C for at least 2 weeks. The values of k_D determined at various starting concentrations of O_2 (within the range from 100 to 320 μM) remain practically unchanged (not shown). This means that molecular oxygen does not participate, at least directly, in the process of HEM degradation. Most likely, lipid peroxy radicals are not involved in HEM degradation

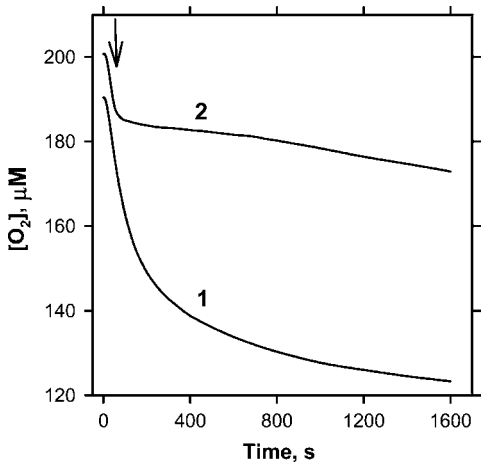

Figure 7. Effect of HPMC on the kinetics of oxidation of 5 mM preoxidized ML ([LOOH] = 0.25 mM) catalyzed by 50 nM metMB at pH 5.0: 1, without HPMC; 2, 1 μM HPMC was added in 40 s after the addition of metMB.

either. **Figure 7** presents the results of two parallel runs with metMB as a catalyst. The rate of catalytic oxidation drops with time rather quickly (plot 1), evidently due to HEM degradation. In the second run (plot 2) 1 μM HPMC was added 30 s after metMB addition, when R_{OX} reached the maximum value of 360 nM s^{-1}. HPMC caused the inhibition of the chain process, and a pronounced induction period was observed. The induction period is completed by nearly 700 s, and the chain oxidation is restarted, even if at a rather low rate. If HPMC, a potent scavenger of peroxy radicals, also prevented the degradation of metMB, R_{OX} by the end of the induction period would be comparable with the R_{OX} of 360 nM s^{-1} recorded at the moment when HPMC was added. In reality, R_{OX} was rather low, nearly $\sim$20 nM s^{-1}, as was determined in the run without HPMC addition (plot 1). This means that purging the reaction mixture from peroxy radicals does not prevent metMB degradation. Similar results were also obtained with Hemin. At first glance, **Figures 7** and **2** are in conflict. Meanwhile, the dramatic difference in the shape of kinetic curves presented in **Figures 2** and **7** may be explained by the difference in the conditions. In the run presented in **Figure 2**, the process occurs with "fresh" ML and at pH 7.4 when metMB is relatively resistant to degradation. Besides, the oxidation of "fresh" ML is accompanied by fast accumulation of LOOH. As a result, the rate of oxidation continues to increase after the moment when HPMC is almost consumed and a minor decrease in [metMB] is compensated by a significant increase in [LOOH]. As for the run presented in **Figure 7**, the oxidation occurs with preoxidized ML at pH 5.0, when metMB is much less stable (see **Figure 6**). As a result, significant reduction of [metMB] is not compensated by a minor increase in [LOOH] by the end of the induction period.

Although the behaviors of metMB and Hemin as catalysts of ML oxidation are qualitatively similar, there is a significant quantitative difference between these two HEM (**Table 1**). At physiological pH, 7.4, the activity of Hemin expressed as k_{IN} exceeds that of metMB by nearly 2 orders of magnitude. At acidic pH, the difference in k_{IN} between metMB and Hemin was less significant; whereas k_{IN} for Hemin is practically independent of pH, k_{IN} for metMB significantly grows when pH decreases. Such a behavior of metMB correlates best with the following mechanism of catalysis by metMB suggested in the literature (*7*, *14*, *15*). Unlike Hemin, metMB, as such, is not a catalyst of lipid peroxidation. The experimentally observed catalysis by metMB is actually caused by a product of metMB denaturation (destruction of a heme−protein adduct), which is a genuine catalyst. Most likely, this is hematin, an analogue of Hemin. As for the pH dependence of the catalytic activity of metMB, this likely explains the observation that the rate of metMB denaturation increases when the pH reduces (*11*, *17*). It should be noted that the mechanism of metMB denaturation is far from clear.

Table 2. Calculation of k_{IN} from Simulated [O_2] Traces at Various Reagent Concentrations by Using Equations 3 and 4[a]

[LH], mM	[LOOH], mM	[HemI], nM	$(R_{OX})_{MAX}$, nM s^{-1}	R_{IN}, nM s^{-1}	k_{IN}, M^{-1} s^{-1}
5	0.1	50	391	24.4	4870
5	0.2	50	552	48.8	4880
5	0.2	100	783	98.2	4910
5	0.3	50	677	73.4	4890
5	0.2	20	348	19.4	4850
10	0.2	20	699	19.6	4890
10	0.1	20	493	9.72	4860
2	0.1	20	98.5	9.70	4850
5	0.1	20	247	9.76	4880

[a] Simulations were performed on the basis of **Scheme 2** at $k_3 = k_{3A} = 1 \times 10^4$ and $k_4 = 0.01$ in all cases.

Table 3. Correlation of Simulated Values of k_{IN} with Rate Constants for Reactions 3 and 3A[a]

k_3	k_{3A}	$(R_{OX})_{MAX}$, nM s^{-1}	R_{IN}, nM s^{-1}	k_{IN}, M^{-1} s^{-1}
20000	20000	493	38.9	9722
10000	10000	345	19.4	4761
5000	5000	242	9.37	2343
2000	2000	150	3.60	900
20000	2000	213	7.26	1815
2000	20000	200	6.40	1600

[a] Simulations were performed on the basis of **Scheme 2**. In all cases $k_4 = 0.01$, [LH] = 5 mM, [LOOH] = 0.2 mM, and [HemI] = 20 nM.

Kinetic Computer Simulations. The simulations were conducted on the basis of **Scheme 2**. All of the rate constants employed in simulations for the reactions of the first and second order are expressed in s^{-1} and M^{-1} s^{-1}, respectively; all of the rates of chemical reactions are given in nM s^{-1}. In all of the simulations the following parameters are used: $k_1 = 500$, $2k_2 = 1 \times 10^6$; [O_2] = 0.2 mM. The simulations allow all of the experimental kinetic curves as well as the observed concentration effects to be imitated. In particular, it was possible to imitate the experimentally observed plots of R_{MAX} versus [Hemin] and [LH] for the case of the oxidation of "fresh", nonoxidized ML, although containing a very small concentration of LOOH (**Figure 3**). These concentration plots may be expressed by a power function: $R_{MAX} \sim [HEM]^A$ and $R_{MAX} \sim [LH]^B$. For both experimental and simulated plots, $A < 1$ and $B > 1$. Simulations also confirm the above-mentioned observations that the values of k_{IN} calculated from the starting values of R_{OX} for the oxidation of preoxidized ML do not depend on reagent concentrations (**Table 2**).

The above presented data suggest that the computer simulations based on Scheme 2 are able to describe the observed experimentally kinetic regularities typical of ML oxidation catalyzed by HEM. This gives us a chance to apply this approach to elucidate some other kinetic regularities. **Table 3** shows how the rate constant for elementary reactions 3 and 3A correlates with the "experimental" value of k_{IN} (the latter was calculated from simulated kinetic curves as has been done with real kinetic curves). It is seen that when $k_3 = k_{3A}$, the calculated value of k_{IN} equals nearly half of k_3 (k_{3A}). In the case when $k_3 \neq k_{3A}$

Table 4. Effect of Values of Rate Constants for Reactions 3 and 4 on Kinetics of the Oxidation As Simulated on the Basis of **Scheme 2**[a]

k_3	k_4	$(R_{OX})_{MAX}$, nM s^{-1}	f
2000	0.01	117	5.59
3000	0.01	159	10.47
5000	0.01	238	23.6
10000	0.01	420	74.1
20000	0.01	775	241
2000	0.005	173	25.2
2000	0.002	351	261
2000	0.001	612	1628

[a] In all cases $k_3 = k_{3A}$, [LH] = 10 mM, [LOOH] = 0.01 mM, $[HemI]_0$ = 50 nM.

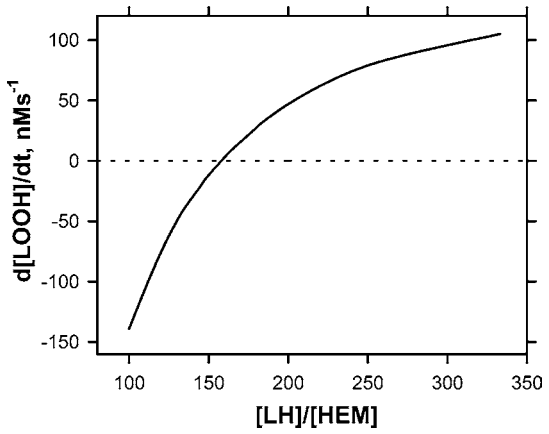

Figure 8. Inversion (going from prooxidation to antioxidation) as simulated on the basis of **Scheme 2**. Parameters used for simulation are $k_3 = k_{3A} = 400$, $k_4 = 0.001$, and [LH] = 2 mM; starting value of [LOOH] is 100 μM. The variation of the [LH]/[HEM] ratio was carried out by variation of HEM concentration. The values of d[LOOH]/dt are related to the moment of 30 s after HEM addition.

("asymmetry" of reactivity), k_{IN} has an intermediate value. As expected, the increase of HEM resistance to degradation (decrease of k_4) results in the significant growth of $(R_{OX})_{MAX}$ and especially of the regeneration coefficient f (**Table 4**).

Figure 8 shows how the character of change in [LOOH] depends on the ratio of reagent concentrations, [LH]/HEM]. This simulation was undertaken in connection with several works (*5*, *16*) reporting that, at a certain [LH]/[HEM] ratio, the inversion from prooxidation to antioxidation was observed. By antioxidation those authors meant the situation when the addition of HEM resulted in the reduction of LOOH concentration, in contrast to its growth in the prooxidation mode. As **Figure 8** suggests, the accumulation of LOOH gives way to its consumption at [LH]/[HEM] $\leq$ 160. The calculated position of the inversion point changes with the variation of rate constants and the starting concentration of LOOH. The physical meaning of the inversion is evident; this follows from **Scheme 2** and the general theory of chain free radical oxidation (see ref *2* and references cited therein). Reactions 3 and 3A play two roles at the same time: the destruction of LOOH and the generation of active free radicals initiating chain oxidation. The rates of both processes are equal. This further depends on the length of kinetics of the chain, υ. At $\upsilon > 1$, LOOH is accumulated; when $\upsilon < 1$, LOOH concentration decreases. The situation when $\upsilon = 1$ corresponds to the inversion point.

Concluding Remarks. A new quantitative approach to the elucidation of the efficacy of iron heme complexes as a catalyst of lipid peroxidation has been suggested in this work. The key point of this approach is the determination of the rate of free radical generation (initiation), R_{IN}, which can be calculated at any moment of time directly from the rate of oxidation, R_{OX}. The HEM efficacy is characterized by two independent parameters: initiating activity and resistance to degradation. The initiation occurs due to the interaction of HEM with ML hydroperoxide. The initiating capability is characterized by the rate constant for this reaction, k_{IN}. A simple and reliable method to determine k_{IN} has been elaborated; this is based on the use of preoxidized ML containing a known concentration of LOOH. This is the first time that it has been shown that the catalytic activity of HEM is reduced progressively in the course of ML oxidation due to HEM degradation. Two ways to characterize HEM resistance to degradation have been suggested. The first one is based on determining the effective rate constant k_D characterizing the decrease in R_{IN} during the starting stage of the process. The second, more general, way is the measurement of the number of active free radicals that can be generated by one molecule of HEM throughout the process. A kinetic scheme adequately describing the process under consideration and the corresponding computer model, which allows for simulating both kinetic curves and kinetic regularities, have been suggested in this work. Along with other things, the computer simulation makes it possible to predict the behavior of the system under study in variable circumstances. In particular, the simulations have supplied quantitative explanations for the inversion effect (going from prooxidation to inhibition during lipid oxidation induced by metMB).

The developed approach has been applied to study the catalytic capability of metMB and its protein-free analogue Hemin. Both HEM were found to be very effective catalysts; even at nanomolar concentrations, they cause ML oxidation at a detectable rate. At physiological pH, 7.4, the activity of Hemin exceeds that of metMB by almost 2 orders of magnitude (**Table 1**). At acidic pH, the difference in activity decreases due to a significant growth of k_{IN} for metMB when pH decreases, in contrast to Hemin. Most likely, Hemin has the highest catalytic activity among other HEM as was recently observed in the course of preliminary studies of series of synthetic analogues of Hemin; any modification of the Hemin molecule resulted in a decrease in catalytic activity as compared with Hemin itself (results will be published elsewhere). The value of f was varied within the range of 40−150 depending on reagent concentrations and pH (**Table 1**), which evidence the effective regeneration of catalytically active forms of HEM. It has been first shown that the catalytic activity of the HEM studied drops rather rapidly with time, evidently due to HEM degradation.

The data obtained in this work in combination with the observations reported in the literature give insight into the behavior of MB under variable conditions. In living muscles, MB exists basically in the reduced form (Fe^{2+}), which is catalytically inactive; a relative concentration of the potentially active oxidized form, metMB, is rather low. The latter is provided due to the action of special enzymes (*37*). Besides, in living muscles the concentration of peroxides participating in active free radical generation is commonly very low. In contrast, the concentration of chain-breaking antioxidants, first of all of vitamin E, is high enough to block almost completely the chain lipid peroxidation. The mentioned factors make MB catalytically inactive and thus nontoxic under normal physiological conditions. The situation changes dramatically when we deal with muscles under post-mortem conditions, that is, with meat foods. In meat, the concentration of metMB progressively increases and the pH becomes more acidic (*8*, *38*, *39*). Both factors as well as the progressive consumption of chain-breaking

antioxidants are favorable for exhibiting the activity by MB (more exactly, metMB) as a catalyst of lipid peroxidation. In the course of time, lipid peroxidation becomes a self-accelerating process, which results in the fast accumulation of LOOH and other toxic products. These processes are considered as the main reason for spoilage of muscle foods under storage conditions. An even more favorable situation for metMB to act as a catalyst of lipid peroxidation takes place in stomach. MetMB comes into the stomach along with the meat consumed. Two factors, a highly acidic pH in the stomach and the fact that lipids consumed are partly oxidized due to previous processing, can cause the explosive lipid peroxidation. This highly undesirable process may be retarded by chain-breaking antioxidants. It is currently believed that this mechanism is responsible for the beneficial effects of natural products such as fruits, vegetables, red wine, tea and coffee extracts, and chocolate, which are rich in polyphenols that are known as the most potent chain-breaking antioxidants (*4*, *5*, *11*).

ABBREVIATIONS USED

HEM, iron heme complexes; MB, myoglobin; metMB, metmyoglobin; HB, hemoglobin; ML, methyl linoleate; LOOH, lipid hydroperoxide; HPMC, 6-hydroxy-2,2,5,7,8-pentamethylbenzochroman; $LO_2^{\bullet}$, lipid peroxy radical; R_{OX}, rate of oxidation; R_{IN}, rate of initiation (free radical generation); k_{IN}, effective rate constant of initiation due to reaction of HEM with LOOH; k_D, rate constant characterizing HEM degradation; t_{IND}, induction period; *f*, coefficient of HEM regeneration (a number of catalytic cycles).

LITERATURE CITED

(1) Baron, C. P.; Andersen, H. J. Myoglobin-induced lipid oxidation. *J. Agric. Food Chem.* **2002**, *50*, 3887−3897.

(2) Jeney, V.; Balla, J.; Yachie, A.; Varga, Z.; Gregory, M.; Vercellotti, G. M.; Eaton, J. W.; Balla, G. Pro-oxidant and cytotoxic effects of circulating heme. *Blood* **2002**, *100*, 879−887.

(3) Reeder, B. J.; Wilson, M. T. Hemoglobin and myoglobin associated oxidative stress: from molecular mechanisms to disease states. *Curr. Med. Chem.* **2005**, *12*, 2741−2751.

(4) Lapidot, T.; Granit, R.; Kanner, J. Lipid hydroperoxidase activity of myoglobin and phenolic antioxidants in simulated gastric fluid. *J. Agric. Food Chem.* **2005**, *53*, 3391−3396.

(5) Kanner, J.; Lapidot, T. The stomach as a bioreactor: dietary lipid peroxidation in the gastric and fluid and the effects of plant-derived antioxidants. *Free Radical Biol. Med.* **2001**, *31*, 1388−1395.

(6) Omedo-Sale, F.; Monti, D.; Olliaro, P.; Taramelli, D. Prooxidant activity of β-hematin (synthetic malaria pigment) in arachidonic acid micelles and phospholipid large unilamellar vesicles. *Biochem. Pharmacol.* **2001**, *61*, 999−1009.

(7) Baron, C. P.; Skibsted, L. H.; Andersen, H. J. Peroxidation of linoleate at physiological pH: hemichrome formation by substrate binding protects against metmyoglobin activation by hydrogen peroxide. *Free Radical Biol. Med.* **2000**, *28*, 549−558.

(8) Faustman, C.; Cassen, R. G. The biochemical basis for discoloration in fresh meat: a review. *J. Muscle Foods* **1990**, *1*, 217−243.

(9) Tang, J.; Faustman, C.; Hoagland, T. A.; Mancini, R. A.; Seyfert, M.; Hunt, M. C. Interactions between mitochondrial lipid oxidation and oxymyoglobin oxidation and the effects of vitamin E. *J. Agric. Food Chem.* **2005**, *53*, 6073−6079.

(10) Grunwald, E. W.; Richards, M. P. Mechanisms of heme protein-mediated lipid oxidation using hemoglobin and myoglobin variants in raw and heated washed muscle. *J. Agric. Food Chem.* **2006**, *54*, 8271−8280.

(11) Vulcain, E.; Goupy, P.; Caris-Veyrat, C.; Dangles, O. Inhibition of the metmyoglobin-induced peroxidation of linoleic acid by dietary antioxidants: action in the aqueous vs. lipid phase. *Free Radical Res.* **2005**, *39*, 547−563.

(12) Lapidot, T.; Granit, R.; Kanner, J. Lipid peroxidation by "free" iron ions and myoglobin as affected by dietary antioxidants in simulated gastric fluids. *J. Agric. Food Chem.* **2005**, *53*, 3383−3390.

(13) Richards, M. P.; Dettmann, M. A.; Grunwald, E. W. Prooxidative characteristics of trout hemoglobin and myoglobin: a role for released heme in oxidation of lipids. *J. Agric. Food Chem.* **2005**, *53*, 10231−10238.

(14) Baron, C. P.; Skibsted, L. H.; Andersen, H. J. Concentration effects in myoglobin-catalyzed peroxidation of linoleate. *J. Agric. Food Chem.* **2002**, *50*, 883−888.

(15) Grunwald, E. W.; Richards, M. P. Studies with myoglobin variants indicate that released hemin is the primary promoter of lipid oxidation in washed fish muscle. *J. Agric. Food Chem.* **2006**, *54*, 4452−4460.

(16) Richards, M. P; Hultin, H. O. Effect of pH on lipid oxidation using trout hemolysate as a catalyst: a possible role for deoxyhemoglobin. *J. Agric. Food Chem.* **2000**, *48*, 3141−3147.

(17) Reeder, B. J.; Wilson, M. T. The effects of pH on the mechanism of hydrogen peroxide and lipid hydroperoxide consumption by myoglobin. A role for the protonated ferryl species. *Free Radical Biol. Med.* **2001**, *30*, 1311−1318.

(18) Kelman, D. J.; DeGray, J. A.; Mason, R. P. Reaction of myoglobin with hydrogen peroxide forms a peroxyl radical which oxidizes substrates. *J. Biol. Chem.* **1994**, *269*, 7458−7463.

(19) Maiorino, M.; Ursini, F.; Cadenas, E. Reactivity of metmyoglobin towards phospholipid hydroperoxides. *Free Radical Biol. Med.* **1994**, *16*, 661−667.

(20) Aoshima, H.; Yoshida, Y.; Taniguichi, H. Reaction between lipid hydroperoxide and hemoglobin studied by a spectrophotometric and a spin trapping method. *Agric. Biol. Chem.* **1986**, *50*, 1777−1783.

(21) Rao, S. I.; Wilks, A.; Hamberg, M.; de Montellano, P. R. O. The lipoxygenase activity of myoglobin oxidation of linoleic acid by the ferryl oxygen. *J. Biol. Chem.* **1994**, *269*, 7210−7216.

(22) Hogg, N.; Rice-Evans, C.; Darley-Usmar, V. M.; Wilson, M. T.; Paganga, G.; Bourne, L. The role of lipid hydroperoxides in the myoglobin-dependent oxidation of LDL. *Arch. Biochem. Biophys.* **1994**, *314*, 39−44.

(23) Baron, C. P.; Skibsted, L. H.; Andersen, H. J. Prooxidative activity of myoglobin species in linoleic acid emulsions. *J. Agric. Food Chem.* **1997**, *45*, 1704−1710.

(24) Roginsky, V. A.; Barsukova, T. K. Kinetics of the oxidation of hydroquinones by molecular oxygen. Effect of SOD. *J. Chem. Soc., Perkin Trans. 2* **2000**, 1575−1582.

(25) Roginsky, V. Chain-breaking antioxidant activity of natural polyphenols as determined during the chain oxidation of methyl linoleate in Triton X-100 micelles. *Arch. Biochem. Biophys.* **2002**, *414*, 261−270.

(26) Loshadkin, D.; Roginsky, V.; Pliss, E. Substituted *p*-hydroquinones as a chain-breaking antioxidant during the oxidation of styrene. *Int. J. Chem. Kinet.* **2002**, *34*, 162−171.

(27) Roginsky, V. A.; Utkin, I. A. The initiating capability of lipid hydroperoxides and kinetics of lipid peroxidation. *Kinet. Catal.* **1991**, *32*, 732−739 (translated from Russian).

(28) Howard, J. A.; Ingold, K. U. Absolute rate constants for hydrocarbone autoxidation. VI. Alkyl aromatic and hydrocarbons. *Can. J. Chem.* **1967**, *45*, 793−806.

(29) Roginsky, V. A. Kinetics of oxidation of polyunsaturated fatty acid esters inhibited by substituted phenols. *Kinet. Catal.* **1990**, *31*, 475−481 (translated from Russian).

(30) Cosgrove, J. P.; Church, D. F.; Pryor, W. A. The kinetics of the autoxidation of polyunsaturated fatty acids. *Lipids* **1987**, *22*, 299−304.

(31) Barclay, L. R. C.; Locke, S. J.; Macneil, J. M. The autoxidation of unsaturated lipids in micells. Synergism of inhibitors vitamin C and E. *Can. J. Chem.* **1983**, *61*, 1288−1290.

(32) Barclay, L. R. C.; Locke, S. J.; Macneil, J. M.; Burton, G. W.; Ingold, K. U. Autoxidation of micelles and model membranes. Quantitative kinetic measurements can be made by using either water-soluble or lipid-soluble initiators with water-soluble or lipid-soluble chain-breaking antioxidants. *J. Am. Chem. Soc*. **1984**, *106*, 2479−2481.
(33) Porter, N. A. Mechanism for the autoxidation of polyunsaturated lipids. *Acc. Chem. Res*. **1986**, *19*, 262−268.
(34) Yamamoto, Y.; Niki, N.; Kamija, Y. Oxidation of lipids. III. Oxidation of methyl linoleate in solution. *Lipids* **1982**, *17*, 870−871.
(35) Burton, G. W.; Doba, T.; Gabe, E. J.; Hughes, L.; Lee, F. L.; Prasad, L.; Ingold, K. U. Autoxidation of biological molecules. 4. Maximizing the antioxidant activity of phenols. *J. Am. Chem. Soc.* **1985**, *107*, 7053−7065.
(36) Nakumura, T.; Maeda, H. A. Simple assay for lipid hydroperoxides based on triphenylphosphine oxidation and high performance liquid chromatography. *Lipids* **1991**, *26*, 765−768.
(37) Arihara, K.; Cassens, R. G.; Greaser, M. L.; Lauchansley, J. B.; Mozdziak, P. E. Localization of metmyoglobin reducing enzyme (NADH-cyt. b5 reductase) system components in bovine skeletal muscle. *Meat Sci*. **1995**, *39*, 205−213.
(38) Kanner, J. Oxidative processes in meat and meat products: quality implication. *Meat Sci.* **1994**, *36*, 169−189.
(39) Baron, C. P.; Andersen, H. J. Myoglobin-induced lipid oxidation: a review. *J. Agric. Food Chem*. **2002**, *50*, 3887−3897.

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