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ARTICLE

Kinetic Studies of Lipid Oxidation Induced by Hemoglobin Measured by Consumption of Dissolved Oxygen in a Liposome Model System

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The effect of hemoglobin (Hb) and lipid concentration, pH, temperature, and different antioxidants on heme-mediated lipid oxidation of liposomes from marine phospholipids was studied. The rate of lipid oxidation was measured by consumption of dissolved oxygen. Heme-mediated lipid oxidation at different Hb and lipid concentrations was modeled by Michaelis−Menten kinetics. The maximum rate (V_{max}) for the reaction with cod and bovine Hb as a pro-oxidant was 66.2 ± 3.4 and 56.6 ± 3.4 μM/min, respectively. The Michaelis−Menten constant (K_m) for the reaction with cod and bovine Hb was 0.67 ± 0.09 and 1.2 ± 0.2 μM, respectively. V_{max} for the relationship between the oxygen uptake rate and lipid concentration was 43.2 ± 1.5 μM/min, while the K_m was 0.93 ± 0.14 mg/mL. The effect of the temperature followed Arrhenius kinetics, and there was no significant difference in activation energy between cod and bovine Hb. The rate of lipid oxidation induced by bovine Hb was highest around pH 6. Ethylenediaminetetraacetic acid (EDTA) had no significant effect on heme-mediated lipid oxidation, but α-tocopherol and astaxanthin worked well as antioxidants. Kinetic differences were found between iron and Hb as pro-oxidants, and the efficacy of the antioxidants depended upon the pro-oxidant in the system.

KEYWORDS: Lipid oxidation; hemoglobin; n-3 PUFAs; EDTA; α-tocopherol; astaxanthin

INTRODUCTION

Lipid oxidation is an important factor for quality loss during processing and storage of food, leading to changes in taste, texture, appearance, and nutritional profile, limiting the shelf life, even in food with a low fat content (*1*−*3*).

Marine lipids contain a high amount of the n-3 polyunsaturated fatty acids [mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)], which are known to have beneficial health effects (*4*). There is great interest in the food industry to use n-3 fatty acids from marine sources in functional foods, but the high susceptibility to oxidation limits their use in processed food.

Transition metals, together with heme pigments, including myoglobin (Mb) and hemoglobin (Hb), have the ability to promote lipid oxidation (*5*). Hb is the main pigment of red blood cells (*6*) and is made up of four polypeptide chains, each containing one heme group, with the latter containing an iron atom inside the heme ring (*7*). Several studies have shown that Hb is an effective promoter of lipid oxidation (*7*−*10*). Hemolysate (ruptured erythrocytes) and methemoglobin (metHb) induced lipid oxidation in soybean phosphatidylcholine (PC) liposomes by a higher rate than the same concentration of ferric sulfate and cupric chloride measured by consumption of dissolved oxygen (*11*). The hemin group (porphyrin ring containing Fe^{3+} iron) have also been reported to be more potent than both ferrous and ferric ions in promoting lipid oxidation measured by thiobarbituric acid reactive substances (TBARS) formation (*12*).

It is well-established that the reaction between metHb and lipid hydroperoxides (LOOH) can lead to the formation of hypervalent iron species (*13*, *14*). The activated form of metHb is perferrylhemoglobin, a short-lived species that, in the absence of external sources of electrons, will rapidly transform to ferrylhemoglobin (ferrylHb) (*14*). The ferryl species of Mb and hemoglobin has the ability to abstract a proton from a lipid (LH) to form an alkoxyl radical (LO•), which can lead to the formation of a peroxyl radical (LOO•) in the presence of oxygen (*15*). LOO• can then react with another LH and give rise to further LOOHs and a free radical cycle.

Tocopherols are known as powerful natural chain-breaking antioxidants that have the ability to react with lipid and peroxyl radicals and convert them to more stable, radical, or nonradical products, thereby preventing the substrate from being oxidized (*2*, *16*). Earlier studies have shown that the addition of α-tocopherol to lipid model systems successfully inhibited Mb-mediated lipid oxidation (*14*, *17*).

In the case of metmyoglobin (metMb)-initiated oxidation, it is believed that α-tocopherol reacts with lipid radicals, thus extending the period with a low degree of oxidation. α-Tocopherol also has the ability to stabilize the ferryl species and protect it from degradation (*14*).

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Hb is said to be a photosensitizer and has the ability to absorb energy from light, which is transferred to triplet oxygen to form singlet oxygen. Singlet oxygen ($^1O_2$) can then react with fatty acids and initiate lipid oxidation (*18*). Carotenoids are efficient molecules for $^1O_2$ quenching (*16*), and their $^1O_2$ quenching activity increases as the number of conjugated double bonds increases (*18*). Carotenoids also have the ability to scavenge free radicals, as proposed by Jørgensen and Skibsted (*19*). The four carotenoids studied (astaxanthin, β-carotene, canthaxanthin, and zeaxanthin) suppressed lipid oxidation mediated by metMb in an emulsion of methyl linoleate.

When studying lipid oxidation, antioxidants are often the main focus, while the effects of pro-oxidants are often overlooked (*20*). An improved knowledge about how Hb promotes oxidation and how different factors influence the activity will lead to a better understanding of how heme-mediated lipid oxidation can be minimized.

The objective of this work has been to study how several factors, such as Hb and lipid concentrations, temperature, pH, and different types of antioxidants, influence heme-mediated lipid oxidation. The rate of the dissolved oxygen uptake rate (OUR) by liposomes made from marine phospholipids was used to continuously monitor peroxidation, the initial step in oxidation, of fatty acids. The results were compared to earlier studies on iron-mediated lipid oxidation (*20*−*22*) to investigate any possible differences in the mechanism of inducing lipid oxidation and how this will affect the choice of the best possible antioxidant.

MATERIALS AND METHODS

Chemicals. 2-Morpholinoethanesulfonic acid (MES), triphenylphosphine (TPP), astaxanthin, Hb from bovine blood (lyophilized powder), $FeSO_4$, and $FeCl_3$ were from Sigma-Aldrich. Ethylenediaminetetraacetic acid (EDTA) was from Merck, while D,L-α-tocopherol was from Calbiochem. All chemicals and solvents were of analytical grade.

Phospholipid Isolation. The phospholipids used to make liposomes were isolated from North Atlantic cod (*Gadus morhua*) roe. Before extraction, the cod roe was kept at −40 °C. The extraction of total lipids was performed according to the method of Bligh and Dyer (*23*). Phospholipids were isolated from total lipids using the acetone precipitation method by Kates (*24*) based on the insolubility of phospholipids in cold acetone. A few modifications of storage time and temperature as described by Mozuraityte et al. (*20*) were made. An aliquot of total lipids, 4 g in 8−10 mL of chloroform, was mixed with 200 mL of acetone and placed in the freezer at −20 °C overnight. The acetone was decanted; the precipitates were dissolved in chloroform; and the isolation was repeated 1 more time. The final precipitates (phospholipids) were stored in chloroform under nitrogen at −20 °C until needed. The fatty acid composition of the phospholipids was analyzed by gas chromatography of fatty methyl esters as described by (*25*), and the lipid classes were determined by thin-layer chromatography as described by Mozuraityte et al. (*21*). Isolated phospholipids contained 98 ± 2% of phospholipids, traces of cholesterol, and unknown compounds. The amount of polyunsaturated fatty acids was 41 ± 9%, of which DHA made up 26 ± 5% and EPA made up 11 ± 2% of total fatty acids.

Preparation of Cod Hb. Cod hemolysate was prepared according to the method of Fyhn et al. (*26*), with some modifications as described by Richards and Hultin (*27*), where the blood cells are lysed in 1 mM Tris buffer (pH 8). After centrifugation, the hemolysate was collected in vials (1.8 mL) and stored at −80 °C until needed.

Quantification of the Cod Hb Concentration. The concentration of cod Hb was quantified according to the method adapted from Brown (*28*) as described by Richards and Hultin (*27*). The cod hemolysate bubbled with carbon monoxide gas was scanned from 440 to 400 nm against a blank containing only buffer using a spectrophotometer (Pharmacia Biotech, Ultrospec 200 UV/vis spectrophotometer). Bovine Hb (Sigma) was used to determine a standard curve to quantify the cod Hb concentration.

Preparation of Liposomes. Liposomes were made as described by Mozuraityte et al. (*20*). The dried film of phospholipids was dissolved in 5 mM MES buffer at pH 5.5 to a concentration of 30 mg/mL. The solution was sonicated with a MSE Ultrasonic Disintegrator Mk2 (MSE Scientific Instruments, Sussex, U.K.), and cooling in ice was used to avoid a temperature decrease. A total sonication time of 2.5 min was enough to disperse all phospholipids in buffer. The preparation of liposomes was performed under natural laboratory conditions for all experiments, except for the experiment where the effect of light on the reaction was studied. In the experiment without light, the liposomes were prepared in the dark and the solution was covered with aluminum foil. Liposomes containing different antioxidants or triphenylphosphine (TPP) were also prepared. The liposomes that were prepared with astaxanthin, α-tocopherol, or triphenylphosphine (TPP) were prepared from phospholipids that had been mixed with either astaxanthin, α-tocopherol, or TPP in chloroform and then dried under nitrogen. The residual solvent was completely evaporated under vacuum in a desiccator containing silica gel at room temperature. Concentrations of 5.4 and 54 μM of astaxanthin and tocopherol (in the reaction cell) were used in the experiments where the effects of these antioxidants were studied. These concentrations were chosen with regard to the lipid/antioxidant ratio and the measurability of the experiment. Final concentrations of TPP in the reaction cell of 100 and 200 μM were used in the experiment where the effect of the peroxide scavenger was studied.

A lipid concentration (in the reaction cell) of 6 mg/mL was used in all of the experiments, except for the experiment where the effect of lipid concentration was studied. In this experiment, the OUR was measured in the lipid concentration interval from 0.6 to 15 mg/mL.

Oxidation Experiments. The consumption of dissolved oxygen by liposomes in a closed, stirred, water-jacketed cell was used as a measure of the rate of lipid oxidation. The concentration of dissolved oxygen was measured continuously by a polarographic oxygen electrode (Hansatech Instrument Ltd., Norfolk, U.K.). The electrode was calibrated daily against air-saturated distilled water (30.0 ± 0.2 °C), taking atmospheric pressure into consideration. At intervals, the calibration of the electrode was performed against a solution of hydrogen peroxide (H_2O_2). When the rate of oxygen consumption was measured, the background OUR was observed for 3−5 min. A total of 20 μL of cod or bovine Hb solution was then injected through a capillary opening in the cell to catalyze lipid oxidation in 1 mL of liposome solution (6 mg/mL).

A working solution of bovine Hb (77 μL) was prepared daily by diluting bovine Hb (Sigma) in 5 mM MES buffer at pH 5.5. The working solution was diluted with 5 mM MES buffer to the desired concentrations. A final concentration of 1.55 μM bovine Hb in the reaction cell was used in all of the experiments, except for the experiment where the effect of the Hb concentration was studied. In the latter experiment, concentrations from the concentration interval of 0.078−3.5 μM (Hb concentration in the reaction cell) were used. Cod hemolysate (2.2 μM) was thawed at room temperature, and the desired concentration was prepared by diluting the hemolysate in 5 mM MES buffer at pH 5.5. A Hb concentration of 1.65 μM in the reaction cell was used in all of the experiments, except for the experiment where the effect of the Hb concentration was studied. In the latter experiment, the concentration in the concentration range of 0.02−2.2 μM (Hb concentration in the reaction cell) was used. Both the bovine Hb solution and the MES buffer that were used in the experiment without light were prepared in the dark and covered with aluminum foil. Bovine Hb was used as a pro-oxidant in the experiments where the effects of the lipid concentration, pH, temperature, light, and different antioxidants were studied.

To verify the experimental temperature, the temperature was measured directly in the cell. All of the experiments (except for determination of activation energy) were performed at 30 °C. In the temperature experiment, the OUR was measured at 10, 20, 25, 30, 35, and 40 °C.

The pH of the buffer was adjusted to the desired value by adding 1 M NaOH or 0.5 N HCl. The correct pH was verified after the oxidation experiment. A pH of 5.5 was used in all of the experiments, except for the experiment where the effect of pH was studied. In the pH experiment, pH values in the interval of 2.0−7.2 were used.

Microsoft Excel was used for data processing. Sigma Plot 10.0 was used for statistical analysis and dynamic curve fitting. It was also used to determine the maximum oxidation rate (V_{max}) and Michaelis−Menten constant (K_m) in the experiments where the effects of the Hb concentration and lipid concentration were studied. Curve fitting was used to determine

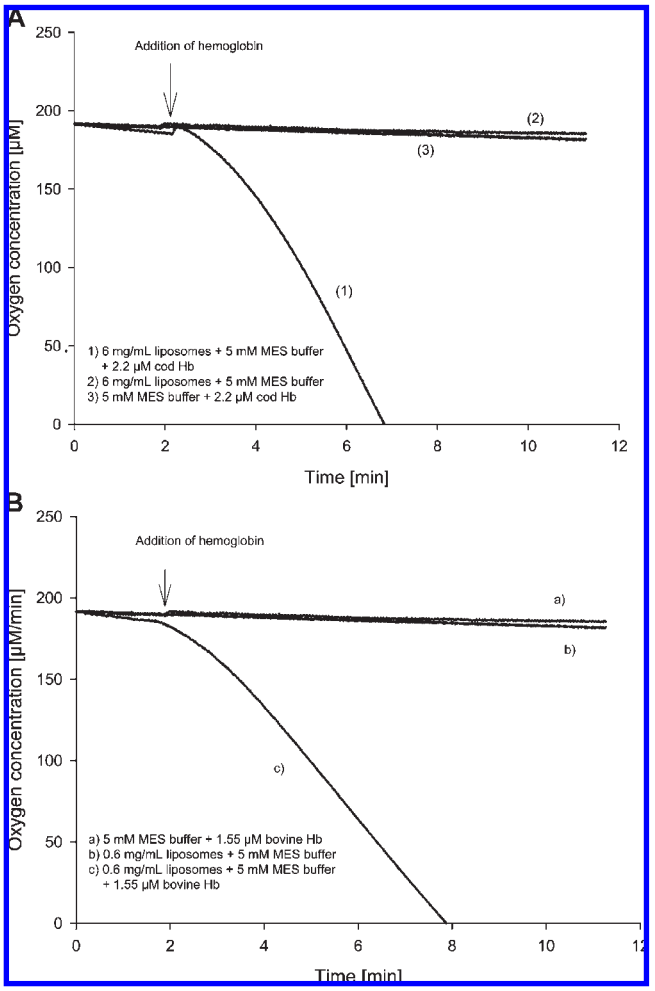

Figure 1. (A) Changes in the dissolved oxygen concentration in a solution of liposomes (6 mg/mL) (1) after injection of cod Hb (2.2 μM). Changes in the dissolved oxygen concentration in a solution of liposomes (6 mg/mL) and MES buffer (5 mM) (2) without injection of Hb (2.2 μM) and in pure MES buffer (3) after injection of cod Hb (2.2 μM). (B) Changes in the dissolved oxygen concentration in a solution of liposomes (6 mg/mL) (1) after injection of bovine Hb (1.55 μM). Changes in the dissolved oxygen concentration in a solution of liposomes (6 mg/mL) and MES buffer (5 mM) (2) without injection of Hb (1.55 μM) and in pure MES buffer (3) after injection of bovine Hb (1.55 μM).

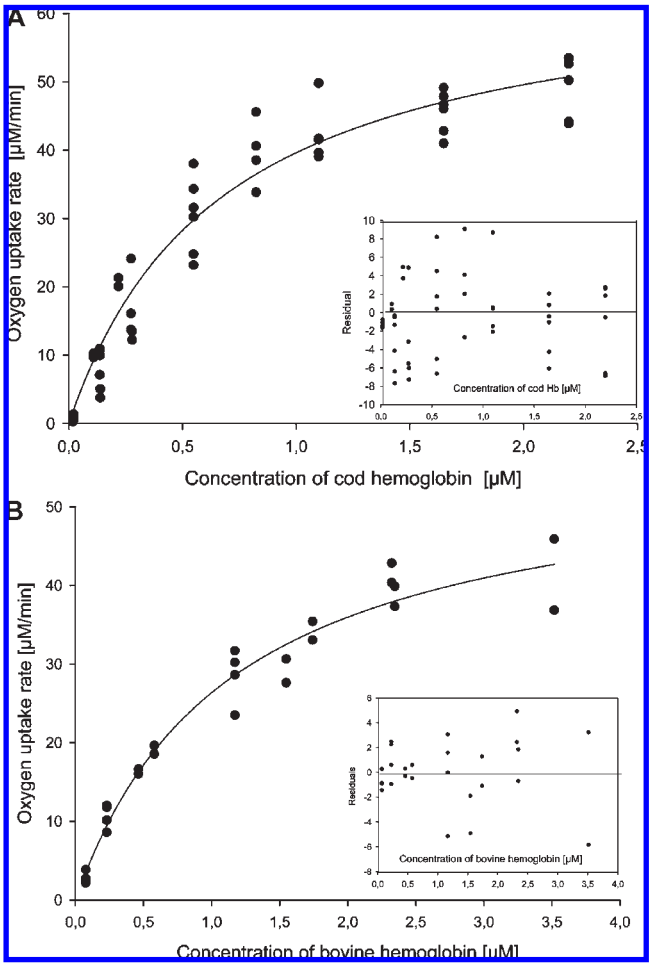

Figure 2. (A) OUR as a function of the cod Hb concentration. The lipid concentration was 6 mg/mL. The temperature was 30 °C, and pH was 5.5. The Sigma Plot was used for dynamic curve fitting of the data points (R = 0.97). The residual plot in the lower right corner shows the relationship between the error in the predicted model and the concentration of cod Hb. (B) OUR as a function of the bovine Hb concentration. The lipid concentration was 6 mg/mL. The temperature was 30 °C, and pH was 5.5. The Sigma Plot was for dynamic curve fitting of the data points (R = 0.98). The residual plot in the lower right corner shows the relationship between the error in the predicted model and the concentration of cod Hb.

at which pH the lipid oxidation rate was highest. Each experiment was performed 3−6 times. The significance level was set at 95% (p = 0.05).

RESULTS AND DISCUSSION

OUR. When cod Hb (2.2 μM) was injected into the reaction cell containing MES buffer and liposomes (6 mg/mL), a fast, accelerating decrease in the oxygen concentration was observed (line a in **Figure 1**), followed by a linear decrease in the oxygen concentration.

The nonlinear decrease after the addition of Hb could be due to the interaction between Hb and pre-existing lipid hydroperoxides and/or that it takes time before steady state is achieved in the system. No significant changes in the oxygen concentration were observed in a solution containing liposomes and buffer (line b in **Figure 1**) nor after injection of Hb to the reaction cell containing only MES buffer (line c in **Figure 1**). An uptake of oxygen was only observed in a system containing Hb, liposomes, and buffer. The same was observed in the experiments were bovine Hb was added to the reaction cell (lines a, b, and c in **Figure 1B**). This indicates that the oxygen uptake is a result of the interaction between the long-chain polyunsaturated fatty acids (LC-PUFA) in liposomes and oxygen mediated by Hb.

The OUR was determined in the linear area, and the linear uptake rate indicates that the OUR is independent of the oxygen concentration and that the oxygen concentration is not rate-determining. The linear OUR also indicates that the reaction system is in equilibrium at steady state.

The decrease in oxygen could also be referred to as the uptake of oxygen, a term that will be used later.

Lipid Oxidation and Hb Concentration. The lipid oxidation rate measured as the OUR as a function of the Hb concentration increased with an increasing Hb concentration. The reaction followed Michaelis−Menten kinetics (panels **A** and **B** of **Figure 2**). The maximum rate (V_{max}) for the reaction with cod and bovine Hb as a pro-oxidant was 66.2 ± 3.4 and 56.6 ± 3.4 μM/min, respectively. The Michaelis−Menten constant (K_m) for the reaction with cod and bovine Hb was 0.67 ± 0.09 and 1.2 ± 0.2 μM, respectively. The residual plot in the lower right corner of **Figures 2A** and **2B** shows the difference between the predicted model and the experimental values as a function of different

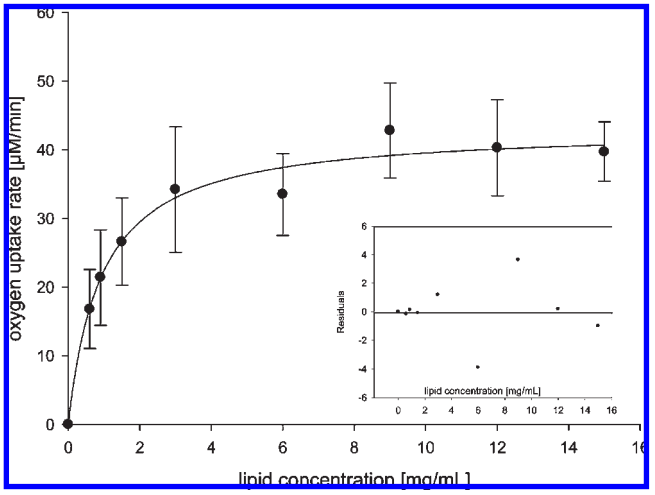

Figure 3. OUR as a function of the lipid concentration. The temperature was 30 °C, and pH was 5.5. Bovine Hb (1.55 μM) was used as pro-oxidant. The Sigma Plot was used for dynamic curve fitting of the data points (R = 0.99). The residual plot in the lower right corner shows the relationship between the error in the predicted model and the lipid concentration.

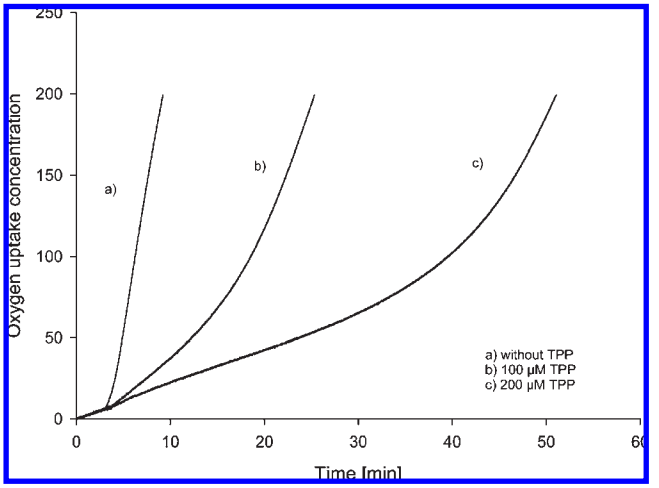

Figure 4. Effect of (a) no, (b) 100 μM, and (c) 200 μM TPP on lipid oxidation induced by bovine Hb. The lipid concentration was 6 mg/mL, while the Hb concentration was 1.55 μM. The temperature was 30 °C, and pH was 5.5.

concentrations of Hb. The points are equally distributed about the x axis, indicating that the selected model gives a good description of the observations.

The lipid oxidation rate (measured as OUR) increases as the concentration of Hb increases. Thus, Hb promotes lipid oxidation in liposomes, an observation that is in agreement with earlier studies performed on Hb as a pro-oxidant (*7*−*10*). Michaelis−Menten kinetics gave a good prediction of the results, and it could be reasonable to assume that, in addition to the catalytic ability of the protein, Hb can also act a substrate in heme-mediated lipid oxidation in liposomes. The ability of Hb to act as a reactant was reported by Undeland et al. (*9*). A doubling of the amount of Hb that they added to the cod mince increased the maximum paint intensity by ∼30% and approximately doubled the maximum intensity of TBARS. This was the case whether the lipid substrate was 0.7% membrane lipids or 0.7% membrane lipids plus 15% oil. These results indicate that Hb limited the extent of oxidation, suggesting that Hb acted as a reactant rather than as a catalyst. The ability of Hb to function as a catalyst is dependent upon the presence of a reducing system regenerating an active reduced form of the catalyst (*29*). Earlier studies have reported that the reaction between Hb and pre-existing lipid hydroperoxides (LOOH) is responsible for the initiation of heme-mediated lipid oxidation (*6*, *30*). Even at very low levels of preformed LOOH, Hb has been the limiting factor for the initiation of lipid oxidation (*31*). When our results and earlier published studies were taken into account, it could be seen that Hb promotes lipid oxidation but that it also limits the extent of oxidation in different model systems.

Lipid Oxidation and Lipid Concentration. The lipid oxidation rate was measured at a lipid concentration interval from 0.6 to 15 mg/mL. The lipid oxidation rate increased with an increasing lipid concentration until it reached a saturation level. The relationship between the lipid concentration and OUR followed Michaelis−Menten kinetics. The maximum OUR was 43.2 ± 1.5 μM/min, while the Michaelis−Menten constant was 0.93 ± 0.14 mg/mL (R^2 = 0.99). Both V_{max} and K_m are determined using curve fitting in Sigma Plot. The residual plot in the lower right corner of **Figure 3** shows the difference between the predicted model and the experimental values as a function of the lipid concentration. The relationship between the OUR and lipid concentration shows that heme-mediated lipid oxidation is dependent upon the lipid concentration. The reason for this could be that metHb needs to be activated by hydrogen peroxide (H_2O_2) or lipid hydroperoxides to be an effective pro-oxidant (*6*), and thus, the reaction will depend upon the concentration of pre-existing lipid hydroperoxides. To determine the importance of pre-existing lipid peroxides on metHb-mediated oxidation, triphenylphosphine (TPP) was added to the phospholipids to reduce the amount of peroxides prior to the formation of liposomes (**Figure 4**). TPP breaks down peroxides to alcohols (*32*) and has been reported to be an effective scavenger of pre-existing lipid peroxides in liposomes (*22*,*32*). Roginsky et al. (*30*) observed an almost complete inhibition of methyl linoleate oxidation induced by Fe−heme complexes. The addition of 100 and 200 μM TPP to the phospholipids before sonication inhibited the initial reaction rate by 94 ± 0.2 and 99 ± 0.2%, respectively. This indicates that LOOH takes part in the reaction and that heme-mediated lipid oxidation in liposomes is dependent upon the presence of pre-existing lipid peroxides. If the reaction between TPP-containing liposomes and Hb continued for more than 20 min, an increase in the OUR was observed. An explanation could be that not all of the pre-existing lipid peroxides are broken down because peroxides can still be measured after the addition of TPP (*22*,*33*). The formation of peroxides produced during liposome preparation could also be an explanation (*22*). After TPP has been consumed, the amount of lipid hydroperoxides will continue to increase and will then be able to interact with Hb and promote lipid oxidation. When Undeland et al. (*9*) added an additional amount of lipid hydroperoxides to washed cod muscle that contained pre-existing LOOH, neither the intensities of paintiness nor the rate of TBARS formation increased. This is in accordance with our observation of the maximum OUR achieved at high lipid concentrations.

Lipid Oxidation and Temperature. As expected, the lipid oxidation rate increased with an increasing temperature. The OUR was measured at different temperatures (10, 20, 25, 30, 35, and 40 °C), and the effect of the temperature on the OUR was analyzed by determining the activation energy (E_a) by the Arrhenius equation (**Figure 5**). The OUR measured in the linear area was used to determine E_a instead of the rate constant because the rate is independent of the oxygen concentration and the change in the amount of non-oxidized lipids was neglectable.

E_a of lipid oxidation induced by cod and bovine Hb was determined to be 47.5 ± 4.2 and 41.1 ± 3.1 kJ/mol, respectively.

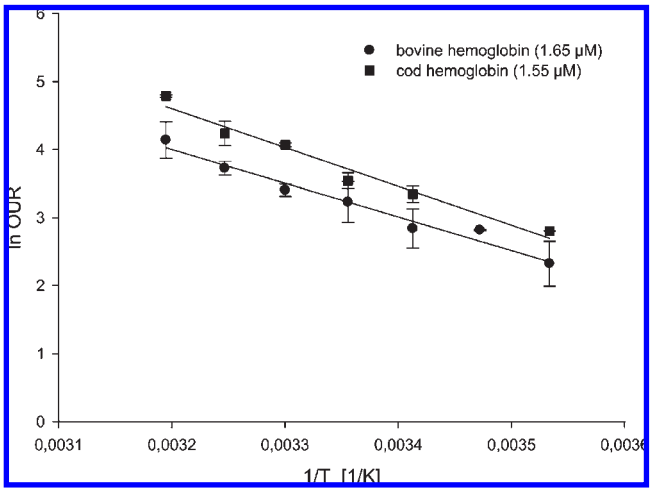

Figure 5. Logarithm of the oxygen uptake rate (ln OUR) as a function of the inverse temperature (1/*T*) for lipid oxidation induced by cod and bovine Hb. The Sigma Plot was used for dynamic curve fitting of the data points [R = 0.98 (cod Hb) and 0.97 (bovine Hb)]. The slope was used to determine the activation energy (E_a). The lipid concentration was 6 mg/mL, and pH was 5.5.

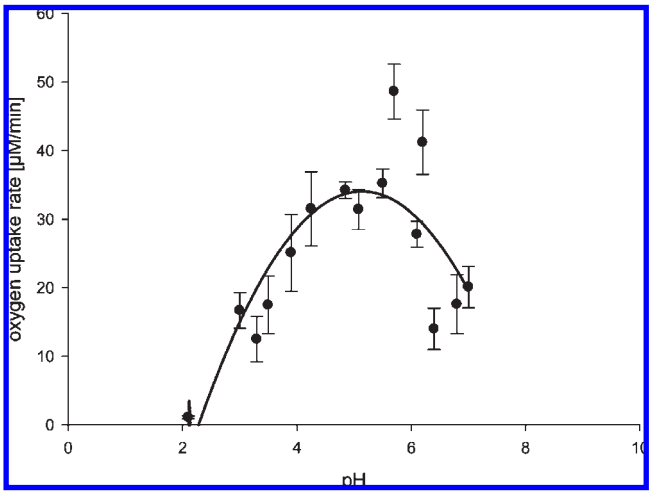

Figure 6. OUR as a function of the pH of the liposome solution. The lipid concentration was 6 mg/mL, and the concentration of cod Hb was 1.65 μM.

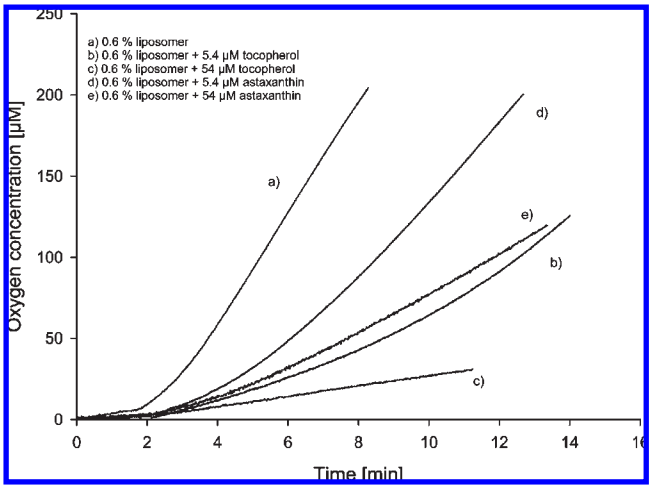

Figure 7. Effect of tocopherol (5.4 and 54 μM) and astaxanthin (5.4 and 54 μM) on lipid oxidation induced by bovine Hb. The lipid concentration was 6 mg/mL; the temperature was 30 °C; and pH was 5.5.

According to the results, there is no significant difference in activation energy between the two types of Hb. Mikkelsen et al. (*34*) studied the ability of horse heart Mb to catalyze lipid oxidation in emulsions made of linoleic acid and Tween 20 by oxygen uptake. Their results gave a more complex temperature profile with two separate linear curves, where the rate increased at temperatures below 23 °C corresponding to an E_a of 28 ± 5 kJ/mol. Kanner and Harel (*35*) reported an activation energy of approximately 105 kJ/mol for lipid oxidation of sarcosomes initiated by metMb. The lipid peroxidation was determined using an oxygen monitor with a Clark electrode. Neither of these results is in agreement with the results in this paper. The variation in activation energy could be due to the difference in the pro-oxidability of Mb and Hb. Another reason could be the difference in model system, difference in substrate and emulsifier, resulting in different rate-limiting steps, and thereby, difference in activation energy.

Lipid Oxidation and pH. The effect of the pH of the liposome solution on heme-mediated lipid oxidation was evaluated in the pH range from 2.0 to 7.0. Bovine Hb was used to initiate lipid oxidation. The curve showing the OUR as a function of pH of the liposome solution was bell-shaped, with a maximum around pH 6 (**Figure 6**).

MetMb is an effective pro-oxidant presence of lipid hydroperoxides (*6*). Reeder and Wilson (*36*) studied the influence of pH on the effectiveness of Mb as a pro-oxidant in the breakdown of lipid hydroperoxides (LOOH). They found that the breakdown of the conjugated diene of LOOH by metMb is strongly pH-dependent. As the pH decreased, the rate constant increased markedly, resulting in an increased first-order rate constant from 9.5×10^{-3} s^{-1} at pH 7 to 2.3×10^{-1} s^{-1} at pH 5. The ability of metMb and metHb to be a more effective pro-oxidant at acidic pH than at physiological pH (7.4) seems to be due to the formation of low-spin hemichrome species (*6*). Baron et al. observed that metMb was not able to initiate linoleate oxidation at physiological pH but rather binds the fatty acid anions to form hemichrome species that cannot be activated by peroxides (*37*). The formation of hemichrome species was also observed during the interaction between Hb and liposomes (*38*). The lack of pro-oxidative activity of metHb at physiological pH could be the reason for the bell-shaped curve that was observed in our studies, because most of the bovine Hb in this experiment was in the oxidized state.

Effect of Light on Heme-Mediated Lipid Oxidation. To study the influence of light on heme-mediated lipid oxidation, an experiment where all of the solutions were made in the dark was performed. The lipid oxidation rate for the reaction performed in the dark was 29.3 ± 6.1 μM/min, while the rate for the reaction performed in the light was 28.1 ± 4.9 μM/min. The results showed that there was no significant difference in OUR between the reaction performed with and without light. Mb and Hb are known as the primary photosensitizers of lipid oxidation in meat. Both Mb and oxymyoglobin (oxyMb) have the ability to absorb light energy, resulting in the formation of metMb and singlet oxygen through a photo-oxidative reaction (*18*). Singlet oxygen can then react directly with double bonds, resulting in the formation of lipid hydroperoxides (LOOH) (*39*). A possible explanation for the lack of influence of light on heme-mediated lipid oxidation could be that most of the Hb used in the experiment was in the oxidized state, thereby leading to a low formation of singlet oxygen.

Effect of Different Types of Antioxidants on Heme-Mediated Lipid Oxidation. EDTA was added to the reaction cell after the injection of pro-oxidant to study the effect of a metal chelator on

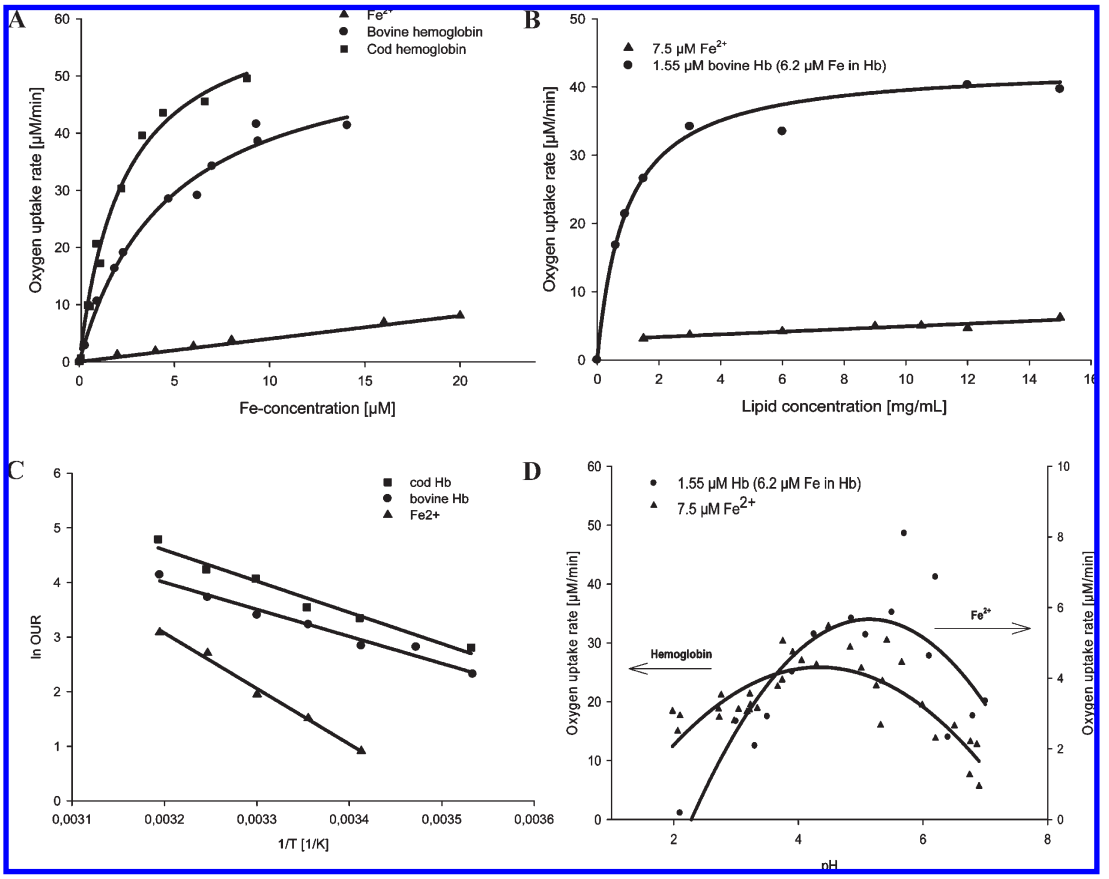

Figure 8. Comparison between the rate of iron-mediated (▲) and heme-mediated (● and ■) lipid oxidation as a function of pro-oxidant and lipid concentrations, temperature, and pH.

heme-mediated lipid oxidation. Because EDTA needs to be deprotonized to be an active metal chelator, the experiment was performed at pH 5.5, a pH value above the pK_a of the ionizable groups [pK 2.0/2.7/6.1/9.5 (*40*)] (*39*). EDTA can either inhibit or promote lipid oxidation induced by free low-molecular-weight iron (*4*). An EDTA/iron ratio higher than 1 inhibits lipid oxidation (*5*). The OURs for the reactions with and without the addition of EDTA were 26.2 ± 3.1 and 31.3 ± 3.2 μM/min, respectively (oxygen uptake curve not shown). The addition of EDTA (2:1) did not inhibit the reaction. This indicates that it is not the free low-molecular-weight iron itself that promote heme-mediated lipid oxidation but rather that the whole intact protein or the heme group is responsible. This is in agreement with the results reported by Richards and Li (*5*), which showed that excess EDTA neither accelerated nor inhibited heme-mediated lipid oxidation in washed cod muscle measured by the peroxide value and TBARS.

Both α-tocopherol (lines a, b, and c in **Figure 7**) and astaxanthin (lines a, d, and e in **Figure 7**) showed a significant inhibitory effect on lipid oxidation initiated by bovine Hb. An addition of 5.4 and 54 μM tocopherol to the liposomes inhibited the reaction rate by 58.5 ± 8.1 and 94.7 ± 1.0%, respectively. After the addition of Hb, a slower initial rate and a longer induction period could be observed in comparison to the initial rate and induction period for liposomes without tocopherol. Studies have shown that the initiation of oxidation of liposomes induced by metHb takes place by radicals formed in the reaction between lipid hydroperoxides in the liposomes and Hb (*11*). The longer induction period could be due to the ability of tocopherols to scavenge peroxyl radicals and induce a lag phase during which the liposomes are not substantially oxidized (*11*). α-Tocopherol has been reported to have an inhibitory effect on lipid oxidation in an oxyMb/liposome model, both alone and together with ascorbate or β-carotene (*41*, *42*). Incorporation of α-tocopherol into the liposome membrane allowed α-tocopherol to scavenge free radicals produced in the lipid phase and both protect the phosphatidylcholine-based liposomes and delay the oxidation of oxyMb (*41*).Yoshida et al. (*11*) studied the effect of 2,2,5,7,8-pentamethyl-6-chromanol (PMC), a vitamin E analogue, on lipid oxidation in soybean phosphatidylcholine (PC) liposomes induced by metHb. They reported that PMC reduced the oxidation rate markedly and that the length of the induction period increased with an increasing concentration of PMC. As mentioned earlier, the initiation of heme-mediated lipid oxidation leads to the formation of lipid radicals responsible for oxidation. Published results (*17*, *41*) indicate that vitamin E delays heme-mediated lipid oxidation by radical scavenging, and these could explain the inhibition effect on lipid oxidation by α-tocopherol in our system.

The addition of 5.4 and 54 μM astaxanthin to the phospholipids before the preparation of liposomes inhibited the lipid oxidation rate by 25.5 ± 6.3 and 62.2 ± 3.9%, respectively. A longer lag phase was also observed in the liposome solution containing astaxanthin. However, the length of the lag phase was shorter than the lag phase caused by α-tocopherol. This could be due to different abilities of the molecules to scavenge peroxyl radicals. While tocopherols inhibit lipid oxidation by donating a hydrogen atom, carotenoids seems to trap radicals both at the conjugated polyene chain and in the terminal ring moiety (*43*). Carotenoids are also efficient singlet-oxygen quenchers (*16*). Both Hb and Mb are known photosensitizers of lipid oxidation and can contribute to the formation of singlet oxygen (*18*). As mentioned earlier, light did not have a significant effect on lipid oxidation

induced by bovine Hb. It is therefore more likely that the formation of free radicals is the primary reaction leading to lipid oxidation.

Iron- and Heme-Mediated Lipid Oxidation. Oxidation in food is influenced by the presence of pro-oxidants, such as transition metals and heme pigments (*39*). This paper shows that Hb is an effective promoter of lipid oxidation and that it is important to increase our knowledge on the mechanism of heme-induced oxidation to be able to select the best antioxidant for the system. As mentioned earlier, low-molecular-weight iron also has the ability to promote lipid oxidation (*39*). It is thus of interest to compare our studies on Hb-initiated oxidation with earlier studies performed with low-molecular-weight iron (*20*, *22*).

A large difference in both catalytic activity and kinetics can be observed by comparing iron- and Hb-mediated oxidation (**Figure 8A**). The OURs show that Hb is a better promoter of lipid oxidation than free molecular iron measured at equal iron concentrations, an observation that is in agreement with the results reported by O'Brien (*44*). As shown in this paper, lipid oxidation as a function of the Hb concentration follows Michaelis−Menten kinetics. This is not the case for lipid oxidation induced by low-molecular-weight iron, which follows pseudo-first-order kinetics (**Figure 8A**). Fe^{2+} catalyzes the breakdown of lipid hydroperoxides (LOOH) and thereby promotes lipid oxidation. In the case of metHb, the ability of the protein to catalyze lipid oxidation is dependent upon the presence of LOOH. MetMb-mediated lipid oxidation will thus be dependent upon the concentration of ferrylHb (the product of the reaction between metHb and LOOH), and this could be the reason for the lipid oxidation as a function of the Hb concentration being described by Michaelis−Menten kinetics.

Lipid oxidation induced by Hb at different lipid concentrations follow Michaelis−Menten kinetics, while iron-induced oxidation at different lipid concentrations is described by a linear curve in the measured range (**Figure 8B**). The comparison shows that heme-mediated lipid oxidation is more dependent upon the lipid concentration than oxidation induced by low-molecular-weight iron. The reason for this could be that metHb needs to be in contact with either H_2O_2 or lipid hydroperoxides to undergo activation to its catalytic form (*13*). This could be an explanation for heme-mediated oxidation being more dependent upon the lipid concentration than iron-catalyzed oxidation. One should also take into account that there could be a possibility for a dependence of the lipid concentration at very low iron concentrations. However, very low oxidation rates make this difficult to measure.

Figure 8C shows the Arrhenius curve for the oxidation induced by cod Hb, bovine Hb, and Fe^{2+}. There is a significant difference in the slope between the curve for Hb-induced oxidation compared to the curve for iron-mediated oxidation, resulting in a higher activation energy for the latter [60−86 kJ/mol (*20*)]. This indicates that the two reactions have different rate-determining steps.

Both the lipid oxidation rate as a function of pH catalyzed by iron or Hb can be described as a bell-shaped curve, but the pH optimum is different (**Figure 8D**). Mozuraityte et al. (*20*) observed a pH optimum around 4.5−5.5 for iron-mediated oxidation, while the pH optimum for heme-mediated oxidation found in this paper is around 6.

The effect of different antioxidants on lipid oxidation induced by Hb and iron was compared. An addition of astaxanthin (5.4 and 54 μM) to the liposomes inhibited both heme- and iron-mediated oxidation (results not shown). The addition of EDTA neither accelerated nor promoted heme-mediated lipid oxidation but had a significant inhibitory effect when low-molecular-weight iron (Fe^{2+} and Fe^{3+}) was used as a pro-oxidant (*4*, *22*). Caffeic acid, a natural phenolic antioxidant, inhibited heme-mediated lipid oxidation but enhanced the rate of iron-mediated lipid oxidation (*45*) (**Table 1**). The study with EDTA and caffeic acid gives an indication that different antioxidants could have a different influence on lipid oxidation depending upon the type of pro-oxidants and model system. In choosing the best antioxidant for a system, it is therefore important to take into account the type of pro-oxidant.

Table 1. Effect of EDTA, Caffeic Acid, α-Tocopherol, and Astaxanthin on Heme- and Iron-Mediated Lipid Oxidation of LC-PUFAs in Liposomes[a]

antioxidant	Hb	iron (Fe^{2+})
EDTA	0	+
caffeic acid	+	−
α-tocopherol	+	
astaxanthin	+	+

[a] An antioxidative effect is marked by +, while a pro-oxidative effect is marked by −. No significant effect is marked by 0.

Both an increase in Hb and lipid concentration resulted in an increased lipid oxidation rate. The effect of the temperature was used to determine the activation energy, and there were no significant difference in the activation energy between cod and bovine Hb. A maximum oxidation rate was found at pH 6. EDTA had no significant effect on the lipid oxidation induced by Hb, while α-tocopherol and astaxanthin worked well as antioxidants. The results were compared to earlier studies on iron-mediated lipid oxidation, and it showed that the different factors studied affected the two systems in different ways.

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