JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effect of pH on Hemoglobin-Catalyzed Lipid Oxidation in Cod Muscle Membranes in Vitro and in Situ

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The effect of pH and hemoglobin on oxidation of the microsomal lipids of cod was determined in isolated microsomes and in washed cod muscle. An increase of hemoglobin concentration from 0.5 to 15 μM accelerated lipid oxidation in both systems. In cod microsomes the rate of lipid oxidation increased in the order pH 6.8 $\gg$ pH 7.6 > pH 8.4 > pH 6.0 > pH 3.5. However, in washed cod muscle a decrease of pH from 7.8 to 6.8 greatly increased the lag phase and decreased the rate of lipid oxidation. A further decrease in pH to 3.5 decreased the lag phase and increased the rate of lipid oxidation further. A decrease of pH from 7.6 to 6.4 greatly reduced the affinity of hemoglobin for oxygen. Formation of methemoglobin due to autoxidation occurred more rapidly at pH 6.0 than at pH 7.5. Structural changes of the isolated microsomal membranes could be the reason for the unexpected slow lipid oxidation in microsomes at pH 6.0 and below.

KEYWORDS: Cod; hemoglobin; phospholipids; lipid oxidation; protein oxidation; membranes; oxidation

INTRODUCTION

Deterioration in flavor, color, texture, nutritive value, and safety can arise from lipid oxidation in muscle-based foods, especially those from dark-muscle fish (*1*). Some possible endogenous catalysts of fish muscle lipid oxidation are hemoglobin (Hb), myoglobin, low molecular weight (LMW) transition metal complexes, and lipoxygenases (*2*). The useful shelf life of fatty fish, especially during frozen storage, is limited by quality loss caused by lipid oxidation. Frozen lean fish is less susceptible to oxidation. However, recent investigations showed a rapid and extensive lipid oxidation catalyzed by hemoglobin in washed cod muscle, which has a low fat content (*3*). Adding fish triacylglycerols to washed cod muscle did not enhance the rate of lipid oxidation (*4*). Thus, the different susceptibilities to lipid oxidation of fatty and lean fish could not be solely related to total fat content.

The two major groups of muscle lipids are phospholipids and triacylglycerols. Phospholipids give structure and fluidity to membranes, and their levels are relatively constant in fish muscle at 0.5−1.5%. On the contrary, triacylglycerol levels vary widely with species and environmental conditions, and the total fat content in fish muscle can vary from 0.5 to 30% (*5*). The triacylglycerols are found in adipose tissues and integrated into muscle tissue both intra- and intercellularly. There is evidence that membrane lipids are primary substrates in lipid oxidation (*6*). This could be due to the high content of highly unsaturated fatty acids of their phospholipids, the close contact of the phospholipids with catalysts of lipid oxidation located in the membranes, and their large surface area exposed to the aqueous phase of muscle cells (*7*).

A previous study compared lipid oxidation and loss of sulfhydryl groups in cod sarcoplasmic reticulum in two systems of low molecular weight complexed iron (*8*). Both polyunsaturated lipids and sulfhydryls are highly susceptible to free radical oxidations. One was a nonenzymic system with ascorbate as a reducing agent, and the other used NADH and the membrane ferric iron reducing enzyme system. Both lipid and protein oxidations of the membrane differed with the different catalysts. The nonenzymic ascorbate system produced very rapid lipid oxidation but a low extent of oxidation compared to the NADH-driven enzyme system, which had an initial longer lag phase than the nonenzymic system. An interesting observation was that the enzymic system oxidized sulfhydryl groups inaccessible to reagents without detergent disruption of the membranes more rapidly than the nonenzymic system. These data were interpreted to mean that the enzymic oxidizing system was able to oxidize components in the interior of the membrane, whereas the nonenzymic system acted most efficiently at the surface.

It is important to study the oxidation of the membrane components by hemoglobin, which we now believe is the major catalyst (possibly with myoglobin) of lipid oxdation in fish. Hemoglobin could have an important role on the different rates of lipid oxidation between lean and dark-muscle fish. Dark-muscle fish species have higher hemoglobin levels than lean fish (*9*). Hemoglobin was the predominant heme protein in mackerel light muscle (6 μmol of hemoglobin/kg) and whole trout muscle (11 μmol of hemoglobin/kg). In mackerel dark

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10.1021/jf0403890 CCC: $30.25
Published on Web 03/29/2005

muscle there was more myoglobin (342 μmol of myoglobin/kg) than hemoglobin (159 μmol of hemoglobin/kg) on a molar basis, although there were more heme monomers contributed by the tetrameric hemoglobin compared to the monomeric myoglobin (*10*). Cod fillets typically contain 1−2 μmol of hemoglobin/kg. Hemoglobin can promote lipid oxidation in fish muscle by means of various pathways. Oxyhemoglobin [HbFe(II)O_2] can be autoxidized to methemoglobin [HbFe(III)] with production of the superoxide anion radical ($O_2^{\bullet -}$). The latter can be converted to hydrogen peroxide (H_2O_2) via a dismutation reaction. The H_2O_2 in turn can produce the hydroxyl radical in the presence of Fe(II). The hypervalent ferryl hemoglobin radical [•HbFe(IV)═O] generated by the reaction of methemoglobin with hydrogen peroxide can initiate lipid oxidation by abstraction of an H atom from a polyunsaturated fatty acid (*11*). Oxygenation and autoxidation of hemoglobin have been found to be inversely related (*12*, *13*). A significant increase of deoxyhemoglobin with pH reduction has been detected in hemoglobin from rainbow trout (*13*, *14*). A pseudolipoxygenase activity has been described for both myoglobin and hemoglobin (*15*). A reduction of pH in post-mortem muscle has an important effect on the ability of hemoglobin to catalyze lipid peroxidation (*13*).

Lipid peroxidation catalyzed by trout hemoglobin was found to increase when the pH was lowered from neutrality to 6.0, and levels of deoxyhemoglobin and methemoglobin were higher at pH 6.0 than at pH >7 (*13*). The affinities for oxygen of hemoglobins from different species of fish, poultry, and beef at pH values below or at neutrality may be different (*16*, *17*). Factors affecting oxy/deoxygenation of heme proteins during post-mortem periods may be crucial to understanding lipid oxidation and fish rancidity development.

The objective of the present study was to investigate the effect of pH and hemoglobin concentration on lipid oxidation promoted by cod hemoglobin on two model systems rich in phospholipids, that is, isolated microsomal membranes from cod muscle and washed cod muscle itself. The stability of cod hemoglobin as a function of pH was also determined by studying oxygenation and autoxidation. Values of pH were chosen that represented the range of values to which muscle might be exposed under a variety of processing conditions around neutrality. The acidic pH (3.5) is used in a new process for isolating fish proteins from low-value muscle tissue raw materials (*18*). Oxidation problems occur readily at this pH.

MATERIALS AND METHODS

Chemicals. Bovine hemoglobin, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer, sodium heparin, tris[hydroxymethyl]aminomethane (Tris), streptomycin sulfate, bovine serum albumin, trichloroacetic acid (TCA), 1,1,3,3-tetraethoxypropane, and reduced glutathione were purchased from Sigma (St. Louis, MO). All other chemicals used were of analytical grade.

Fish Supply. Atlantic cod fish (*Gadus morhua*) was obtained from day boats out of Gloucester, MA. The fish was transported in ice to the laboratory where it was filleted, skinned, and minced. All red muscle was removed. The initial pH values of the cod ranged from 6.5 to 6.7.

Preparation of Cod Hemolysate. The cod blood was collected from cod frames. The frames were obtained after filleting in the rigor state. The fish blood was taken from the caudal vein after the tail of the fish frame was cut off. Blood was collected with a transfer glass pipet rinsed with 150 mM NaCl with sodium heparin solution (30 units/mL), and it was immediately mixed with ~1 volume of the saline sodium heparin solution. Hemolysate was prepared by following the modification by Richards and Hultin (*13*) of the procedure of Fyhn et al. (*19*). Hemolysate was stored at − 80 °C and was thawed just before it was used.

Quantification of Hemoglobin. Hemoglobin (Hb) levels were quantified with modifications of the method by Brown (*20*). The hemolysate was diluted in 50 mM Tris, pH 8.6. Diluted hemolysate was bubbled with carbon monoxide gas (Matheson Gas, Gloucester, MA) for 20 s. Diluted hemolysate was deposited into a 1.5 mL disposable cuvette, and ~1 mg of sodium dithionite was added and mixed. The sample was then scanned from 440 to 400 nm against a blank that contained only buffer, using a a double-beam spectrophotometer model U-3110 (Hitachi Instruments, Inc., San Jose, CA). The peak absorbance was recorded, and the absorbance at the peak minus the absorbance at the valley was calculated and taken as the concentration of Hb. A standard curve was made with Hb from bovine blood.

Cod Hemoglobin Oxygenation. The affinity of Hb for oxygen was determined by scanning Hb solutions in 50 mM sodium phosphate, from 640 to 500 nm, using a double-beam spectrophotometer model U-3110 (Hitachi Instruments, Inc.). The Hb concentration was 1 μM, the temperature, 6−8 °C, and the pH range, 6.0−8.0. The blank contained only buffer. The absorbance at the peak (574 nm) minus the absorbance at the valley (560 nm) was recorded. The difference in absorbance was a measure of oxygenation.

Cod Hemoglobin Autoxidation. Cod hemolysate was diluted to 10 μM in 0.12 M potassium chloride and 5 mM histidine buffer. Changes in spectra from 360 to 640 nm were determined at pH 6.0 and 7.5 and 6−8 °C using a double-beam spectrophotometer model U-3110 (Hitachi Instruments, Inc.). The blank contained only buffer.

Isolation of Cod Membrane. The method of McDonald and Hultin (*21*) was modified for isolating cod membrane. Four volumes of chilled 0.1 M HEPES buffer (pH 7.5) was added to the minced cod muscle and homogenized using a Polytron PT 10-35 (Brinkman Instruments, Westbury, NY) for 30 s. The homogenate was adjusted to pH 7.5 and centrifuged at 10000*g* for 20 min at 5−10 °C in a Beckman L8-55M ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). The supernatant was centrifuged at 130000*g* for 30 min at 5−10 °C. The resulting sediment was suspended in chilled 0.12 M KCl and 5 mM histidine (pH 6.8) buffer with a Potter homogenizer and stored at −80 °C for no longer than 1 week.

Microsomal Model System. The isolated membrane suspended in 0.12 M potassium chloride and 5 mM histidine (pH 6.8) was diluted to a final concentration of 0.7 mg of membrane protein/mL with the same buffer solution. Oxidation was initiated by the addition of hemoglobin to a final concentration of 0.5−15 μM. Incubation was carried out in 25 mL Erlenmeyer flasks in a shaking water bath at 6 °C. The pH was adjusted to the desired value before hemoglobin was added, and it was rechecked after the addition of hemoglobin. In the controls hemoglobin was replaced by 1 mM Tris (pH 8.0) buffer. Oxidation was followed by measurement of thiobarbituric acid reactive substances (TBARS) and by loss of sulfhydryl groups (see below).

Membrane aggregation was studied at pH 6.0 and 7.0 as a function of time by measuring the soluble proteins in the supernatant after centrifugation for 10 min at 1500−2000*g* at 6−8 °C in a tabletop centrifuge (IEC clinical centrifuge, International Equipment Co., Needham, MA).

Washed Cod Muscle Model System. The washed cod muscle was prepared according to a modification of the procedure of Richards and Hultin (*13*). Cod light muscle was minced through a plate with orifices of 4.7 mm in a KitchenAid mincer (KitchenAid Inc., St. Joseph, MI). The mince was washed twice in distilled deionized water at a 1:3 ratio of muscle/water (w/w) by stirring with a plastic rod for 2 min. After the mixture had been stirred for 15 min, it was dewatered on a fiberglass screen. The retentate was mixed with 50 mM sodium phosphate, 0.12 M potassium choride, and 5 mM histidine buffer to pH 5.8, 6.8, or 7.8 at the same 1:3 ratio. The mixture was homogenizated for 1 min using the Polytron PT 10-35. The mixture stood for 15 min and then was centrifuged at 15000*g* for 20 min at 4 °C to separate solid and liquid phases. The washed cod was frozen at −80 °C until used. The muscle was thawed in a sealed plastic bag under running cold water. After the pH was adjusted to the desired value with HCl or NAOH, the moisture was measured. Streptomycin sulfate (200 ppm) was added to inhibit microbial growth. The final moisture was adjusted to 88%. A volume of hemoglobin stock was added to a final concentration of 3 μmol/kg of washed cod muscle. In the control the hemoglobin was replaced by

Table 1. Composition of Cod Muscle, Washed Cod Muscle, and Cod Membrane

						dry wt basis		
sample	% water	% protein	% TL[a]	% PL[b]	% PL/TL	% protein	% TL	% PL
cod muscle	81.2 ± 0.1	16.7 ± 0.9	0.94 ± 0.03	0.78 ± 0.04	83.1 ± 4.2	94.6 ± 5.1	5.3 ± 0.2	4.4 ± 0.2
washed cod muscle[c]	86.7 ± 0.1	13.3 ± 0.3	0.46 ± 0.05	0.39 ± 0.01	86.0 ± 3.1	96.7 ± 2.3	3.3 ± 0.4	2.9 ± 0.1
cod membrane					97.6 ± 2.6	66.2 ± 1.8	33.7 ± 1.3	33.0 ± 0.9

[a] TL, total lipid. [b] PL, phospholipid. [c] The washed cod sample was prepared at pH 6.8.

distilled water. The samples were stored in capped 125 mL Erlenmeyer flasks with a sample thickness of 6−7 mm at 2 °C.

Thiobarbituric Acid Reactive Substances Analyses. TBARS were determined according to the method of McDonald and Hultin (*21*) in washed cod muscle and the membrane model system. 1,1,3,3-Tetraethoxypropane was used as standard. The data are expressed in terms of nanomoles of malonaldehyde.

Total Sulfhydryl Group Analysis. Total sulfhydryl groups were determined according to the method of Viner et al. (*22*). Reduced glutathione was used as standard.

Determination of Protein Content. Protein content was determined according to the method of Markwell et al. (*23*). Bovine serum albumin was used as standard. Suspended proteins in the membrane preparation were determined in the supernatant after centrifugation at 1500−2000*g* for 10 min.

Determination of Total Lipids. Total lipids were extracted according to the method of Lee et al. (*24*) using a 1:1 (v/v) chloroform/methanol mixture. The total lipid content was obtained by gravimetric analysis after evaporation of the solvent.

Determination of Phospholipid Content. The phospholipid content was estimated by measuring organic phosphorus in the extracted lipid (*3*). Phosphorus content was converted to phospholipid by assuming each g-atom of phosphorus was equivalent to 750 g of phospholipid.

Data Collection. Results with washed cod were obtained on duplicate experiments with duplicate analyses, wheres experiments with the membranes were done three times with single determinations. Results presented are from a single typical experiment.

RESULTS

The composition of cod white muscle, washed cod muscle, and cod membrane is shown in **Table 1**. The yield of isolated membrane was ∼520 mg of membrane (on a dry weight basis) per 100 g of wet muscle. Phospholipid recovery in the membrane preparation was ∼23%. This estimate is based on the amount of phospholipid in the isolated membrane preparation compared to the phospholipid concentration in the original muscle tissue.

Oxygenation and Autoxidation of Cod Hemoglobin. The affinity of cod Hb for oxygen decreased at acidic conditions between pH 6.0 and 8.0 (**Figure 1**), with a strong decrease from pH 7.6 to 6.4. A similar result was obtained with trout hemoglobin (*13*).

Autoxidation of cod Hb was dependent on pH. Cod Hb at pH 6.0 had noticeable spectral changes after 1 h (**Figure 2a**). Spectral shifts were observed with time of incubation in the Soret band as the peak shifted to a shorter wavelength (414 → 406 nm) (**Figure 2a**). By 6 h the visible spectrum of methemoglobin was established. Cod hemoglobin at pH 7.5 had greater stability to oxidation than it did at pH 6 (**Figure 2b**). At pH 7.5 some reduced Hb remained after 48 h, as a positive difference of absorbance between 574 nm (peak) and 560 nm (valley) was observed. However, some autoxidation of hemoglobin to methemoglobin after 48 h was evidenced by the decrease of absorbance at 574 nm and the increase at 630 nm.

Lipid and Protein Oxidation Promoted by Cod Hemoglobin in a Model System of Cod Microsomes. Increasing Hb concentrations between 0.5 and 5 μM reduced the lag phase.

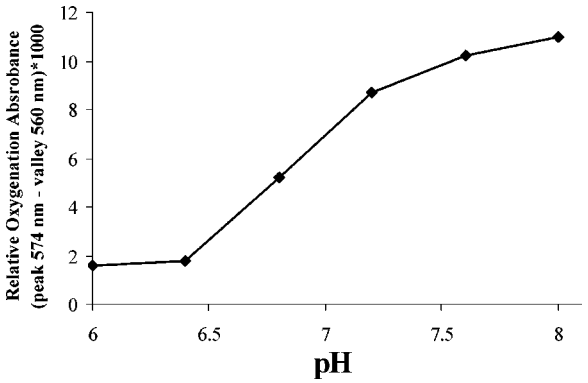

Figure 1. Relative oxygenation of cod Hb in 50 mM sodium phosphate buffer as a function of pH at 6–8 °C. Oxygenation was measured as the difference in absorbance between the peak (574 nm) minus the valley (560 nm) of the Hb spectrum. Hemoglobin that was more oxygenated showed higher differences in absorbance between the peak and valley. Hemoglobin concentration was 1 μM.

Increasing concentrations of Hb increased the maximal amount of lipid oxidation as well as the oxidation rate as measured by TBARS at pH 6.0 (**Figure 3**).

The effect of pH on lipid oxdidation (as TBARS) promoted by 5 μM cod Hb for the microsomal system was determined (**Figure 4**). The shortest lag time was observed in the sample at pH 6.8. The reaction catalyzed by Hb at pH 7.6 had a longer lag time, but the rate of oxidation was similar to that observed at pH 6.8. At pH 6.0 and 8.4, the lag times for the oxidation were longer than they were at pH 6.8 and 7.6, but once started, the oxidation rates appeared to be somewhat faster than they were at pH 6.8 or 7.6. The maximum amount of TBARS produced at pH 6.0 was a little higher than the amount of TBARS produced at 6.8, 7.6, or 8.4. The oxidation at pH 3.5 had the longest lag time and the slowest oxidation rate once it started. However, at the end of 7.5 h, the TBARS values were still increasing and had reached a value similar to that of the sample at pH 6.0.

In another series of experiments, rates of lipid oxidation catalyzed by the Hb were followed at pH 6.0 and 8.0 with and without a prior incubation of the membrane for 30 min at pH 3.5 (**Figure 5**). The rate of oxidation of the membrane was followed at pH 3.5 as a control. The lag phase of the lipid oxidation reaction was shorter at pH 8 than it was at pH 6.0, although the rates of oxidation were similar once the oxidation went into the accelerated phase. The longest lag phase for oxidation occurred at pH 3.5. The samples that were directly evaluated at pH 8.0 or 6.0 showed higher rates of oxidation than did the corresponding samples that had first been exposed to a low pH. The difference between the samples that had been treated at low pH and those that had not was somewhat greater for pH 8 than for pH 6. These results make it clear that not only does pH affect hemoglobin but exposure of the membrane

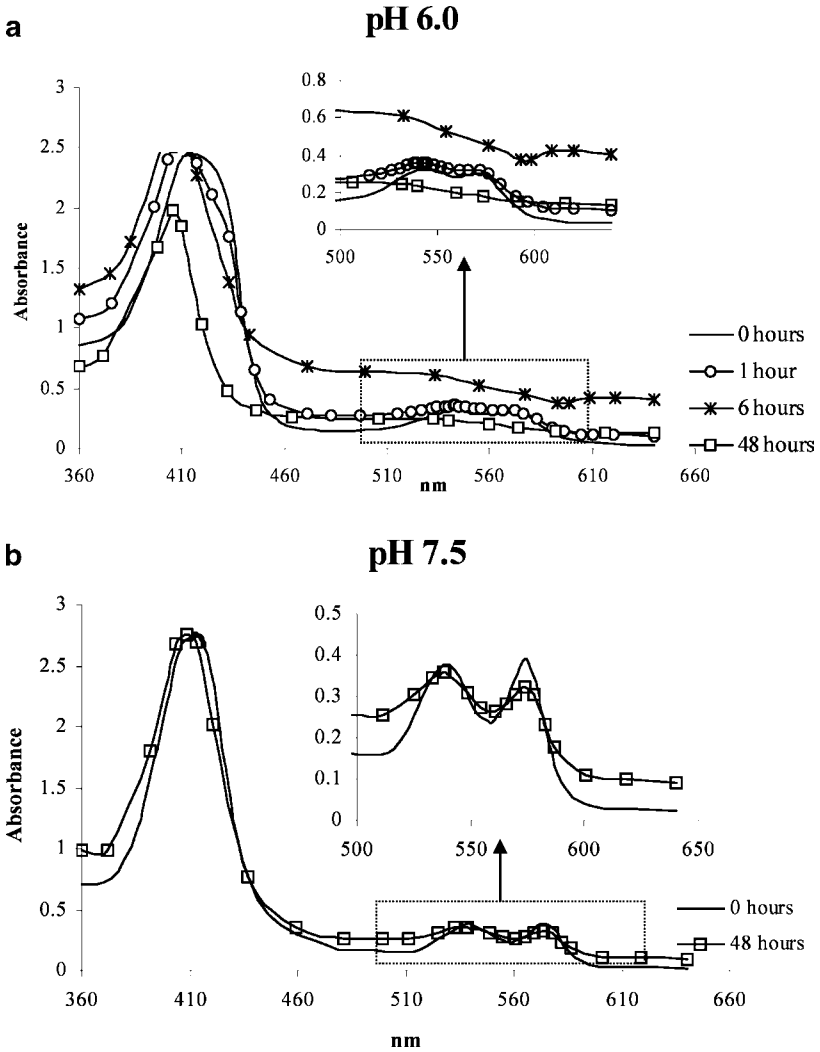

Figure 2. Spectral changes of 10 μM cod Hb in 0.12 M KCl and 5 mM histidine at pH 6.0 (**a**) and 7.5 (**b**) as a function of time. The temperature of storage was 6–8 °C.

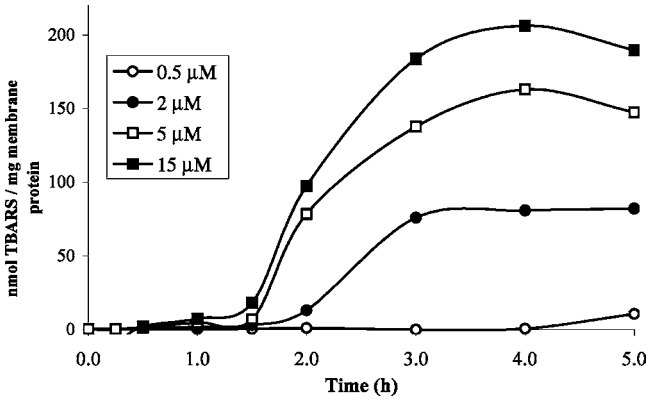

Figure 3. Effect of concentration of cod Hb on lipid oxidation measured as TBARS in the microsomal model system at pH 6.0.

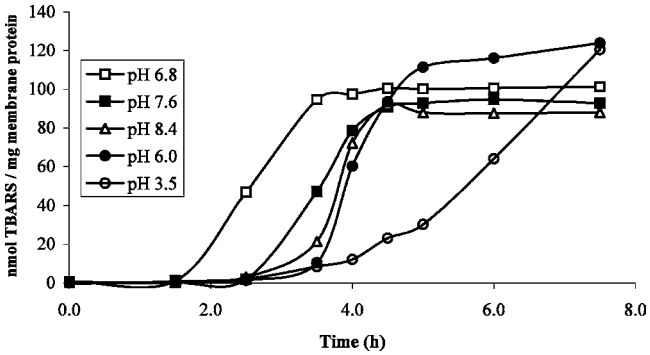

Figure 4. Effect of pH on lipid oxidation mediated by 5 μM cod Hb in a cod microsomal model system. The final membrane concentration was 0.7 mg of membrane protein/mL.

to pH 3.5 induced changes, chemical or physical, in the cod microsomal membrane, rendering it less susceptible to oxidation by Hb.

The effect of pH on the loss of sulfhydryl groups in a suspension of 5 μM Hb and cod microsomal membrane at 0.7 mg of protein/mL is shown in **Figure 6**. The loss of sulfhydryl groups paralleled inversely the production of TBARS shown in **Figure 4**. The loss of sulfhydryl groups was similar at pH 6.0 and 8.4, and this loss occurred shortly after the loss seen at pH 7.6. Sulfhydryl losses at pH 6.8 began by 2 h of incubation as opposed to the lag time of ~4 h for the previous three pH values cited. The content of sulfhydryl groups at pH 3.5 showed a rather rapid drop initially but then stabilized after 1.5 h up to almost 4 h before it also underwent a rapid decrease. It is interesting that the final concentration of sulfhydryl groups in the membrane preparations after exposure to Hb seems to be essentially the same for all of the samples. This may represent some end point that is a reflection of exposed sulfhydryl groups.

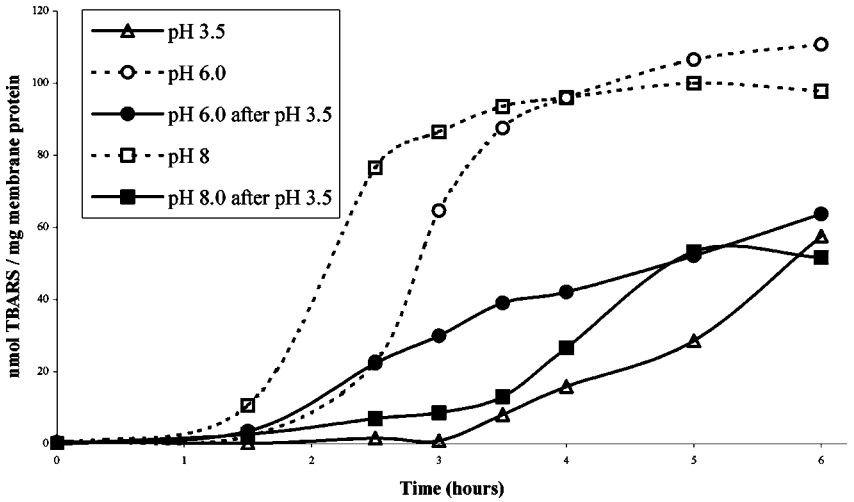

Figure 5. Effect of prior incubation of cod microsomal membranes at pH 3.5 on lipid oxidation promoted by 5 μM cod Hb containing 0.7 mg of protein/mL. Assay for lipid oxidation at each pH was performed with and without a 30 min preincubation at pH 3.5. The control sample at pH 3.5 was directly assayed at that pH.

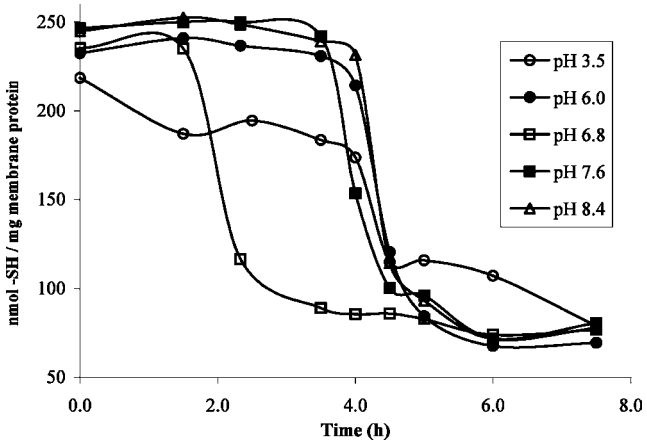

Figure 6. Effect of pH on loss of total sulfhydryl groups mediated by 5 μM cod Hb in a cod microsomal model system as a function of time.

Five micromolar hemoglobin used in this experiment is equivalent to ~0.34 mg of protein/mL. Thus, Hb in the assay system makes up approximately one-third of the total protein. In this system, 0.7 mg of membrane protein/mL contributed ~150 nmol of sulfhydryl groups, whereas the 0.34 mg of Hb contributed ~120 nmol of Hb protein sulfhydryls. In **Figure 6** all of the values went from approximately 240 to 250 nmol of sulfhydryl groups/mg of protein to a value below 100, which indicates that sulfhydryl groups from both the membrane proteins and the Hb were lost. It is not possible at this time to determine how much of the loss came from each of these sources. Although the loss of sulfhydryls at the four higher pH values followed rather closely the TBARS results at the same pH values, the pH 3.5 pattern was different. This may be related to the large loss of sulfhydryl groups occurring at pH 3.5 in the absence of Hb (**Table 2**).

Lipid Oxidation Promoted by Cod Hemoglobin in Washed, Minced Cod Muscle. Lipid oxidation catalyzed by Hb was next studied in a model system of washed, minced cod muscle with added hemoglobin. The primary lipids of the washed cod system are the membrane lipids as there are few storage triacylglycerols in cod white muscle. In these samples, some 86% of the total lipids were phospholipids. Undoubtedly some of the non-phospholipids of the cod muscle were membrane components such as cholesterol and tocopherols. When oxidation was studied

Table 2. Loss of Sulfhydryl Groups of Membranes in the Absence of Hemoglobin in 6 h as a Function of pH

loss[a]	pH 8.4	pH 7.6	PH 6.8	pH 6.0	pH 3.5
nmol of total –SH/mg of membrane protein	7.0	2.1	7.3	12.6	40.9

[a] The initial content of –SH groups was 130 nmol/mg of membrane protein. Loss was calculated by determining –SH content after 6 h and subtracting from the initial value.

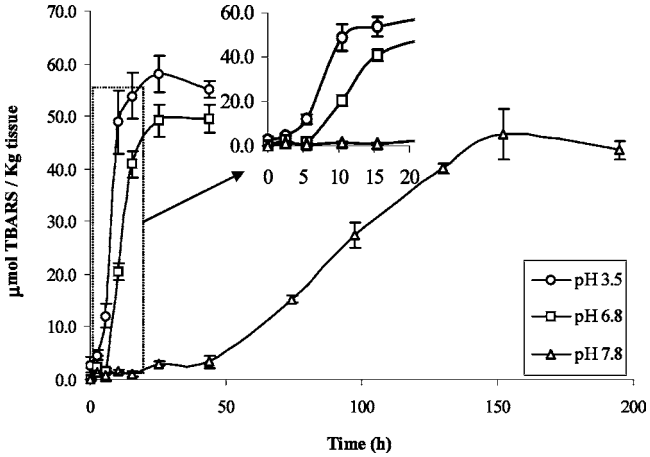

Figure 7. Effect of pH on lipid oxidation mediated by cod Hb in a washed cod muscle model system. The Hb concentration was 3 μmol of hemoglobin/kg of tissue.

in washed cod muscle, the results were quite different from those observed with Hb-catalyzed lipid oxidation of the isolated membrane fractions (**Figure 7**). The oxidation at pH 3.5 had a very short lag phase and a very high rate of oxidation. At pH 6.8 the lag phase was somewhat longer, and the oxidation rate was also somewhat reduced. However, both of these oxidation rates were much faster than was observed at pH 7.8 where the lag phase was ~40 h compared to 6 and 3 h for pH 6.8 and 3.5, respectively. The rate of oxidation was also much slower at pH 7.8 compared to pH 6.8 or 3.5. There was, however, not a great difference in the total amount of TBARS produced after the peak of oxidation was reached. The general relationship of the oxidation rates was similar to what had been seen earlier with the washed cod muscle system using trout Hb (*25*).

Table 3. Soluble/Suspendable Protein at pH 6 and 7

	soluble/suspended protein (mg/mL)	
time (h)	pH 6	pH 7
0.33	0.435	0.481
1	0.465	0.519
2	0.412	0.534
3	0.431	0.584
4	0.260	0.569

Sedimentation Rates of Membranes at pH 6 and 7. It had previously been shown that isolated cod membranes could be easily sedimented at a centrifugal force as low as 1000*g* for 15 min if the pH of the suspending medium was 5 to 3 (*26*). The ready sedimentation at pH 3 occurred without any incubation time. This high sedimentation did not occur at pH $\geq$6. The incubation times of the assays in this study were done over several days. Normally a neutral pH is used to isolate and store the isolated membrane preparations. Therefore, a study was done to compare the aggregation properties of isolated membranes at pH 6 and 7 over several hours at 6 °C. This was done by centrifuging the samples at 1500−2000*g* for 10 min at 6 °C (**Table 3**). Over a 4 h period some 40% of the proteins of the membrane preparation at pH 6 changed sufficiently to be removed at this low centrifugation speed. There was no observable loss in suspended protein under the same conditions at pH 7. Thus, the effect of low pH on the aggregation properties of the membranes are both pH- and time-dependent, occurring rapidly at pH 3 and less so at pH 6. It remains to be seen whether the sedimentation properties of the membrane are related to the changes observed in oxidation of the membrane.

DISCUSSION

Conditions affecting oxygenation of cod hemoglobin and its stability as a function of pH were similar qualitatively to what has been observed with trout Hb (*13*, *27*). The ability of the cod Hb to bind O_2 and its conformational stability were much higher at neutral pH and above than at pH 6.0. There were, nevertheless, some structural changes indicated at pH 7.5 that were greater than those at pH 7 with trout Hb. This indicates that cod Hb is less stable than trout Hb.

Increasing concentrations of cod Hb led to some shortening of the lag phase, some increase in the maximal rate of oxidation, and an increase in the total amount of TBARS produced (**Figure 3**). The increase in maximal reaction rate as well as the total amount of TBARS was roughly proportional to the hemoglobin contents at 2 and 5 μM. However, the relationship lost its proportional aspect at the highest concentration (15 μM) of Hb. The oxidation at 0.5 μM was very low. This approximate stoichiometry of total oxidation produced at low Hb concentrations and a decreasing stoichiometric relationship of total oxidation and catalyst concentration on a molar basis at higher concentrations of Hb had been previously observed with trout Hb and washed cod (*28*). Similar qualitative results were observed with a sarcoplasmic reticular membrane fraction from cod when ADP-Fe^{3+} was tested with an NADH-mediated reduction of the iron by a membrane enzyme (*29*). That is, there was a roughly stoichimetric relationship for the extent of oxidation at low iron concentrations, but this stoichiometry was lost at higher iron concentrations. Thus, it seems that hemoglobin, like low molecular weight complexed iron, functions not only as a catalyst but also as a reactant in the system. The ability of either to function as a catalyst depends on the presence of some reducing system to regenerate an active reduced form of the catalyst.

An important observation in this paper was that the oxidation of lipid in the isolated membrane system was very different from the production of TBARS in the washed, minced cod muscle. As had been found previously with trout Hb, low pH greatly stimulated oxidation in the washed cod model system (*13*, *25*). There was extremely rapid oxidation at pH 3.5 and a still very fast oxidation at pH 6.8. There was then a considerable lag and a slower rate of oxidation at pH 7.8. The oxidation rate of the washed cod system initiated with cod Hb was much faster than the similar rate from trout Hb (*13*).

The washed cod muscle system has proven to be a useful model for studying the effects of pro-oxidants on fish membrane lipid oxidation because it contains little triacylglycerol while maintaining a matrix of protein similar to that present in the original muscle. The washing process removes an efficient low molecular weight, heat-stable antioxidative system that exists in the aqueous phase of cod muscle (*30*). In this study, the Hb-catalyzed lipid oxidation of the isolated membrane fraction had a maximal rate of oxidation at pH 6.8, whereas the slowest rate of oxidation, surprisingly, was found at pH 3.5 (**Figure 4**). These data suggest, therefore, that something occurred at pH 3.5 in the hemoglobin−membrane system that was different in some important respect(s) from what occurred in the membrane lipids of the washed cod muscle. Because the major membrane component of the isolated membranes is the sarcoplasmic reticulum (*31*) and the major membrane component of the cod muscle itself is the sarcoplasmic reticulum, it seems unlikely that the slow rate of oxidation at pH 3.5 was due to a selective isolation of a particular type of membrane for the membrane assay system compared to the membranes normally present in the muscle tissue.

The fact that the maximal rate of oxidation and the shortest lag phase in the hemoglobin−membrane system occurred at a pH (6.8) that was intermediate between two higher and two lower pH values suggests that more than one factor was controlling the rate of lipid oxidation in the isolated membrane system. Because the rate at pH 3.5 was the slowest, it would seem that one of these factors was some modification in the system at low pH which caused the oxidation to be slower than it is at higher pH values. This slow rate at pH 3.5 was observed only in the microsomal system and not in the washed minced cod muscle system, which suggests that it was something specific to the membrane itself which did not happen in the matrix of the muscle protein. The factor that reduced the oxidative activity at a high pH was most likely the reduced pro-oxidative activity of the Hb with increasing pH (*13*). Thus, we conclude that the rate of oxidation as a function of pH over the range of 5.5−8.4 was determined by these two factors, that is, the decrease in Hb-catalyzed activity at high pH versus the decrease in the susceptibility of the membrane to oxidation at low pH. Thus, the slower oxidation rates at pH values above 6.8, that is, pH 7.6 and 8.4, would be caused by the lower pro-oxidative activity of Hb at these alkaline pH values. The longer lag phase that occurred at pH 6 and 3.5 could be due to some modification of the membrane making it more resistant to oxidation. This modification may have been expressed as an aggregation of the membrane as was observed on centrifugation (**Table 3**). The different changes balanced out closely at pH 6.0 and 8.4, giving almost identical oxidation rates.

The loss of sulfhydryl groups was possibly affected by similar conditions because sulfhydryls can serve as antioxidants in lipid oxidation. A loss of sulfhydryl groups appeared first at pH 6.8. This was followed after some period of time by the loss at 7.6 (similar to the increase in TBARS), and this was then followed

by the losses of sulfhydryls at pH 6.0 and 8.4, which were almost identical. The major qualitative difference observed between the production of TBARS as a function of pH and the loss of sulfhydryl groups was at pH 3.5. There was some early loss in sulfhydryl groups at pH 3.5 before losses were observed at any of the other pH values. This was followed by a steady state and finally a decrease at about the same time that the loss of sulfhydryls occurred at pH 6.0, 7.6, and 8.4. Control groups at these various pH values, that is, those in which there was no Hb added to the system, showed that there was a much higher loss of sulfhydryl groups at pH 3.5 than there was at the other pH values (**Table 2**). This instability of sulfhydryl groups at the low pH may have been the cause of the difference in response of the membrane to the loss of sulfhydryl groups at pH 3.5 compared to the rate of lipid oxidation observed at that same pH; that is, the sulfhydryls may have been better free radical scavengers at pH 3.5 than at higher pH values.

It had previously been shown that at pH values of ≤ 5, isolated cod muscle membranes are easily sedimentable at a combined *g* force and time which is much less than the lower *g* force used in the original differential centrifugation to isolate the membranes. This has been attributed to aggregation caused by either fusion of the membranes or a surface-to-surface interaction leading to aggregation (*26*). It seems likely that some type of charge neutralization of the surface of the membrane must happen for either of these mechanisms. Thus, it is tempting to suggest that this type of fusion/aggregation results in physical changes rendering the unsaturated fatty acids less exposed to the aqueous phase in which the Hb would be active. With a longer incubation time (4 h), there seemed to be production of an easily sedimented fraction of the cod muscle membranes during the time period over which the oxidation studies were carried out (**Table 2**). This indicates that it is not just the pH of the medium but also the time of exposure to that medium that results in this "aggregation" of the membranes.

It is possible that other factors also contribute to this decrease in the susceptibility to oxidation at low pH. We observed an increased amount of non-hemoglobin-catalyzed loss of sulfhydryl groups in membranes at pH 3.5 compared to pH ≥ 6.0. It is possible that this greater loss is due to the higher susceptibility of the sulfhydryl groups to conversion to thiyl free radicals at low pH, making them better free radical scavengers. At the same time, the sulfhydryl groups of the membranes could be less effective relatively in the washed, minced muscle, where there is an abundance of protein sulfhydryl groups than in isolated membranes. The sulfhydryl groups of the myofibrillar proteins could exchange with the thiyl radicals of the membrane proteins. This would remove the thiyl radicals from the vicinity of the membrane lipids, thus reducing the susceptibility of these lipids to oxidation.

Whatever the mechanism is for the protection of the membrane lipids against oxidation by low pH, it was at least in part irreversible because incubation at the low pH produced a membrane that was less susceptible to lipid oxidation when the low pH was readjusted upward to the original value (**Figure 5**). Determining the exact mechanism of the low pH protection effect could provide a useful tool for protecting sensitive fish lipids from oxidation during processing and storage.

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Received for review September 8, 2004. Revised manuscript received February 8, 2005. Accepted February 17, 2005. This study was supported by the Cooperative State Research, Extension, Education Service, U.S. Department of Agriculture, Massachusetts Agricultural Experiment Station, under Project MAS00834, by Grant 2002-01659 of the USDA National Research Initiative Competitive Grants Program, by Xunta de Galicia (PGIDTOOAGR20901PR), and by the Spanish Ministry of Science and Technology (PPQ2000-0688-C05-05-03 and Ph.D. grant to M.P.).

JF0403890