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Effect of Low pH on the Susceptibility of Isolated Cod (*Gadus morhua*) Microsomes to Lipid Oxidation

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During the extraction of muscle to produce protein isolates by acid or alkali solubilization, membranes are exposed to abnormally low or high pH. Low but not high pH treatment induces rapid oxidation of membrane phospholipids in the presence of hemoglobin. The goal of this research work was to study the oxidative stability of microsomes under the conditions met during acid solubilization. Isolated microsomes from cod muscle were used as a model system. At pH 5.3 or lower, 99% of isolated cod membranes sedimented at low centrifugation speeds. Isolated membranes that were exposed to pH 3.0 were less susceptible to hemoglobin-mediated lipid oxidation. Cod hemoglobin exposed to pH 3 was rendered less pro-oxidative than the untreated cod hemoglobin. However, when microsomes and hemoglobin were together exposed to low pH, oxidation was promoted. Citric acid and calcium chloride, as well as press juice isolated from cod muscle, were able to inhibit lipid oxidation of microsomal suspensions.

KEYWORDS: Lipid oxidation; acid solubilization; hemoglobin; cod microsomes; press juice from cod; oxidation of cod lipids

1. INTRODUCTION

The phospholipids of muscle tissue are generally considered more susceptible to oxidative deterioration than the neutral triacylglycerols, even though the amount of triacylglycerols might be much higher than the amount of phospholipids present in muscle tissue (*1*–*4*). This is attributed to their high degree of unsaturation (*5*–*7*), as well as to the fact that due to their arrangement in the bilayer, the phospholipids have a very large surface area per unit weight (*8*). The presence of lipid peroxides and pro-oxidants in the membranes, such as Fe-containing proteins, also contributes to their susceptibility to lipid oxidation. It is widely accepted that the oxidation of liposomes or low-density lipoprotein by heme proteins can be initiated by small amounts of lipid hydroperoxides (*9, 10*). Some aspects of lipid oxidation of sarcoplasmic reticular membranes of muscle tissue and of mitochondrial membranes have been studied in detail, and much is known about the factors involved (*11*–*13*).

Methods have been developed to separate proteins from muscle tissue with good functional properties (*14, 15*). The process involves extracting proteins using a high (∼10.5) or a low (∼3) pH to solubilize the muscle proteins and selectively recovering the soluble proteins by isoelectric precipitation (pH ∼5.5) and centrifugation. These large pH shifts can have an impact on both the pro-oxidative properties of hemoglobin (Hb) and the susceptibility of the muscle to lipid oxidation. It was shown that after low pH treatment the washed cod muscle became slightly more susceptible to lipid oxidation, while alkaline treatment slightly protected the muscle from lipid oxidation mediated by trout Hb (*16*). The same authors demonstrated that exposure of trout Hb to low pH increased its pro-oxidative properties. Longer unfolding times and a lower pH led to less refolded Hb and increased pro-oxidative activity. Autoxidation of Hbs was found to occur around pH 7.0 and below but much less at pH 8.0 (*17*). Recent findings indicate that high oxidation state myoglobin species, that is, perferryl- and ferryl-myoglobin, are produced in vivo and are major pro-oxidative candidates in muscle-based foods (*18*). Low pH favors the protonation of the ferryl species, which exhibit great instability and can be considered as possessing a radical-like nature (*19*). Hb subunits were found to autoxidize more rapidly than tetramers (*20*), and Dumoulin et al. (*21*) showed that subunit formation increases an order of magnitude for each unit of pH reduction. Richards and Hultin (*22*) reported that the lag time prior to rancidity of washed cod muscle in a slightly acid environment (pH 6.0) was greatly decreased as compared to pH 7.2, when trout Hb was used as a catalyst. A decrease of pH from 7.6 to 6.4 greatly reduced the affinity of cod Hb for oxygen (*23*). The decrease in oxygen affinity coincided with increased pro-oxidative activity in a washed cod system.

Richards and Hultin (*22*) showed that there was a rapid trout Hb-mediated lipid oxidation of washed cod muscle at pH 3.5, while there was a considerable lag phase and a slower rate of oxidation at pH 7.8. However, in cod microsomes, the rate of Hb-mediated lipid oxidation increased in the order pH 6.8 > >

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pH 7.6 > pH 8.4 > pH 6 > pH 4.5 > pH 3.5 (*23*). In other words, oxidation of membrane lipids is rapid in the intact muscle at pH 3.5, while it is slow in the isolated membranes. Nevertheless, in washed cod muscle, a decrease in pH from 7.6 to 6.4 decreased the lag phase and increased the rate of lipid oxidation. A further decrease in pH to 3.5 decreased the lag phase and increased the rate further. It was postulated that structural changes of the isolated microsomal membranes could be the reason for the unexpected slow lipid oxidation in microsomal preparations at pH 6.0 and below (*23*).

This research work examines some physical and chemical changes of the cod membranes in vitro as a function of pH and the concomitant effect on Hb-mediated, NADH-dependent, and low molecular weight iron-mediated lipid oxidation. Cod microsomes were chosen because of our past experience with this material and the highly unsaturated nature of cod phospholipid fatty acids.

2. MATERIALS AND METHODS

2.1. Materials. Fillets of Atlantic cod (*Gadus morhua*) were purchased from a local fish distributor (J.B. Wright, Gloucester, MA) and transported to the laboratory on ice. Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All reagents were of ACS grade, and all solvents were of high-performance liquid chromatography grade.

2.2. Methods. *2.2.1. Preparation and Quantification of Hemolysate.* Cod frames were severed at the tail section. Blood was collected from the severed vein using a heparin-washed glass micropipette and transferred into heparin solution (containing 30 units of heparin per mL and 150 mM NaCl). The hemolysate was prepared from the blood according to Fyhn et al. (*24*) as modified by Richards and Hultin (*22*), where red blood cells were washed and lysed to release Hb. The Hb levels were quantified spectrophotometrically as described by Brown (*25*), using a model U-3110 double beam spectrophotometer (Hitachi Instruments, Inc., San Jose, CA).

2.2.2. pH Treatment of Hb. A certain volume of the hemolysate was transferred into glass tubes wrapped in aluminum foil to protect them from light and placed on ice. A predetermined volume of 0.4 N HCl (i.e., 0.2–0.3 mL), enough to adjust the pH to 3, was added, and the solution was briefly vortexed. The pH was checked, and after 20 s or 20 min of incubation, it was adjusted to the desired value with 0.4 N NaOH.

2.2.3. Preparation of Microsomal Suspensions (MSs). MSs were prepared by the method of Apgar and Hultin (*26*) with some modifications. The modifications were used to get higher yields of isolated microsomes. Fresh cod fillets were minced twice with a Kitchen Aid mincer (model KSM90, Kitchen Aid Inc., St. Joseph, MI). Four volumes of histidine buffer (0.12 M KCl, 5 mM histidine, pH 7.4) were added to the minced cod muscle. The mixture was then homogenized by a Polytron homogenizer (model PT 10-35, Kinematica AG, Littau, Switzerland) at speed 5 for two bursts of 40 s. The homogenized muscle tissue was centrifuged at 12000*g* for 20 min (Beckman L8-M Ultracentrifuge with TY 19 rotor, Palo Alto, CA). The resultant supernatant fraction was collected and centrifuged again at 100000*g* for 30 min (Beckman L8-M Ultracentrifuge with TY 45 rotor). The sediment was then resuspended in 0.6 M KCl by a Potter–Elvehjem tissue grinder and centrifuged at 100000*g* for 30 min. The resultant sediment was resuspended in histidine buffer (pH 6.8 or 7.4) with the Potter–Elvehjem tissue grinder and used as the MS. The centrifuge was set at 4 °C, and the samples were kept on ice throughout the process. The protein content of the MSs was determined by the method of Markwell et al. (*27*).

2.2.4. pH Effect on Membrane Sedimentation. The isolated membranes, suspended in histidine buffer (5 mM histidine, 0.12 M KCl, pH 7.4), were analyzed for protein content. Then, the pH was adjusted to different values in the range from 6 to 5. The sedimentation of the microsomes was measured as the % of protein remaining in the supernatant after centrifugation of the membrane preparation for 10 min at ~2000*g* at 8 °C in a tabletop centrifuge (IEC Clinical centrifuge, International Equipment Co., Needham, MA).

2.2.5. Effect of pH and Time on Oxidation of MSs. MSs were exposed to pH 3 for different times, ranging from several seconds to 1 h, and then readjusted to pH 6.8 or 7.4 (=treated MS). Hb was also exposed to pH 3 for 20 s or 20 min and then adjusted to pH 6.8 or 7.4 (=treated Hb). The oxidative stability of the treated and untreated suspensions using treated or untreated Hb as a catalyst was studied. Hb was used at a final concentration of 6 μM. The assays contained 0.7 mg membrane protein/mL solution, and the samples were placed in 25 mL Erlenmeyer flasks in a shaking ice–water bath. Oxidation was followed by the production of thiobarbituric acid reactive substances (TBARS), which were measured according to Lemon (*28*) as modified by Richards and Hultin (*22*). Results are expressed in terms of malonaldehyde (MDA) per mg membrane protein. The lag phase was defined as the point on the abscissa (time coordinate) where the extrapolated straight line portion of the oxidation curve intersects.

The effect of pH on the NADH-dependent (enzymic) and iron–ascorbate (nonenzymic)-mediated lipid oxidation of MSs was also investigated. MSs were prepared as described previously and exposed to pH 3 for 1 h before readjustment back to 6.8. The final concentrations in the reaction media of the enzymic oxidation were NADH, 100 μM; ADP, 100 μM; and ferric chloride, 5 or 10 μM, while those of the nonenzymic system were sodium ascorbate, 100 μM; ADP, 100 μM; and ferric chloride, 5 μM. The assays contained 0.7 mg membrane protein/mL solution, and the samples were placed in 25 mL Erlenmeyer flasks in a shaking ice–water bath. Oxidation was followed by measuring the production of TBARS.

2.2.6. Effect of Citric Acid and Calcium Chloride on Oxidation of MSs. MSs were prepared in histidine buffer as described earlier at pH 6.8. Citric acid and calcium chloride at final concentrations of 5 and 10 mM, respectively, were added to the final oxidation assays, followed by the addition of Hb at a final concentration of 6 μM. Then, the samples were either left untreated or exposed to pH 3 for 30 min before readjusting them to 6.8. Oxidation was followed by measuring the production of TBARS.

MSs were also prepared by adding citric acid and calcium chloride (5 and 10 mM final concentration, respectively) in the homogenate of minced cod. The homogenate was incubated for 30 min at pH ~5.4 (i.e., the pH achieved by the addition of citric acid and calcium chloride). Then, the pH was readjusted to 7.4, and membranes were prepared as previously described. Their oxidation at pH 6.8 using untreated Hb (6 μM) as a catalyst was monitored by TBARS formation.

2.2.7. Effect of Press Juice (PJ) on Microsomal Lipid Oxidation. PJ was prepared from minced fresh cod muscle by centrifugation at 22000*g* for 15 h at 4 °C as described by Undeland et al. (*32*) using a Beckman L8-M ultracentrifuge with type 19 Ti rotor (Beckman Coulter Inc., Fullerton, CA). MSs were prepared as described earlier at pH 7.4. PJs at various final dilution ratios as compared to initial (up to 22-fold dilution) and Hb were added to the suspensions. The final protein concentration of the assays was 0.7 mg membrane protein/mL. Hb was used at a concentration of 6 μM. Oxidation was followed by measuring the production of TBARS.

The effect of PJ on the NADH-dependent and iron–ascorbate-mediated lipid oxidation of MSs was also investigated. MSs were prepared as described previously, and PJ at different dilutions (up to 100-fold) was added. The final concentrations in the reaction media of the enzymic oxidation were NADH, 100 μM; ADP, 100 μM; and ferric chloride, 10 μM, while those of the nonenzymic system were sodium ascorbate, 100 μM; ADP, 100 μM; and ferric chloride, 5 μM.

2.2.8. Statistical Analysis. Data were analyzed by analysis of variance using SAS 9.1 (SAS Institute Inc., Cary, NC). Differences between treatment means at the 5% level were determined using the Duncan multiple range test. All treatments were evaluated from three to 10 times with the exception of those of **Figure 7**, which were performed in duplicate. However, the results in the figures are those of duplicate samples with analyses conducted in duplicate, where all of the samples in each experiment were tested at the same time on the same membrane preparation. This was done to reduce variability due to microsomal

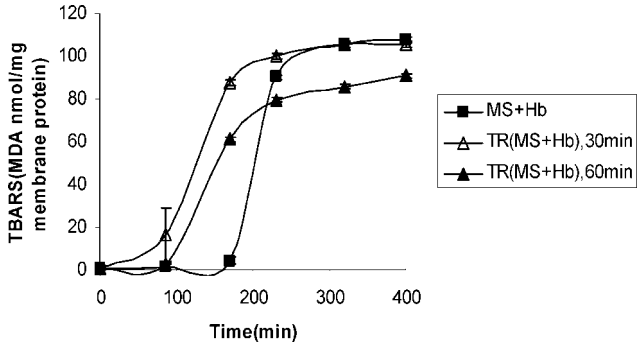

Figure 1. Effect on lipid oxidation of microsomes when membranes and Hb are exposed to pH 3.0 together ($n = 2$). MS + Hb, MS and Hb at pH 7.4. TR (MS + Hb), MS and Hb exposed together to pH 3.0 for 30 or 60 min and then readjusted to pH 7.4. Hb was added at a final concentration of 6 μM.

Table 1. Effect of Treating MSs[a] and Hb Together at pH 3 for 5 or 15 min on the Duration of the Lag Phase of Lipid Oxidation[b]

	duration of lag phase (min)	
experiment	MS	TR (MS + Hb), 5 min, pH 7.4
1	120	130
2	105	85
3	60	110

	MS	TR (MS + Hb), 15 min, pH 6.8
1	40	100
2	50	40

[a] MSs and Hb (6 μM final concentration) were together exposed to pH 3 for 5 or 15 min and then readjusted to pH 6.8 or 7.4. [b] The lag phase is defined as the point on the abscissa (time axis) where the extension of the straight line portion of the oxidation curve intersects.

preparation procedures and to emphasize the effects of the factors being tested. Results are reported as means with standard errors.

3. RESULTS

3.1. Effect of Treating Membranes and/or Hb at pH 3. During the acid solubilization procedure for making protein isolates, the muscle and the Hb are together exposed to pH 3 for a certain amount of time, before being adjusted to pH 5.5 for precipitation. When microsomes and Hb were exposed together to pH 3 and then brought back to higher pH, oxidation was promoted as compared to untreated microsomes (**Figure 1**). The trend is similar to the observation made by Vareltzis and Hultin (*29*) with washed cod oxidation, where oxidation was promoted when the muscle and the Hb were together exposed to pH 3. Exposure of membranes and Hb together at pH 3 for 30 or 60 min decreased the lag phase of oxidation by at least half. However, exposure times of less than 30 min showed that treatment of MS and Hb together at pH 3 could either decrease or increase the duration of the lag phase of the oxidation (**Table 1**).

Separate treatment of the microsomes and Hb at pH 3 for 30 min and 20 s, respectively, rendered the membranes less susceptible to lipid oxidation when assayed at pH 6.8 (**Figure 2a**). Kristinsson (*30*) and Kristinsson and Hultin (*16*) found that the lower the pH and the longer the holding time at low pH, the more misfolded trout Hb became and in turn the more pro-oxidative they became. The reasons for these species differences are not known.

In another experiment, cod Hb was exposed to pH 3.0 for 20 s or 20 min and then readjusted to pH 6.8. Treated Hb (6 μM) was added to untreated MSs, and oxidation assays were run at pH 6.8. **Figure 2b** shows that Hb treated for 20 s or 20 min at pH 3.0 was less reactive as compared to untreated Hb, causing less oxidation of untreated MSs. No significant difference in terms of pro-oxidative activity was found between Hb treated for 20 s and treated for 20 min under these experimental conditions. The direct effect of pH 3 on MSs was done by exposing the microsomes for different times (10 s or 15 or 30 min) and then readjusting to pH 6.8 before adding untreated Hb. Acid treatment significantly reduced lipid oxidation (**Figure 3**). Incubating the MS for 30 min rendered the membranes less susceptible to lipid oxidation as compared to 10 s and 15 min incubation times.

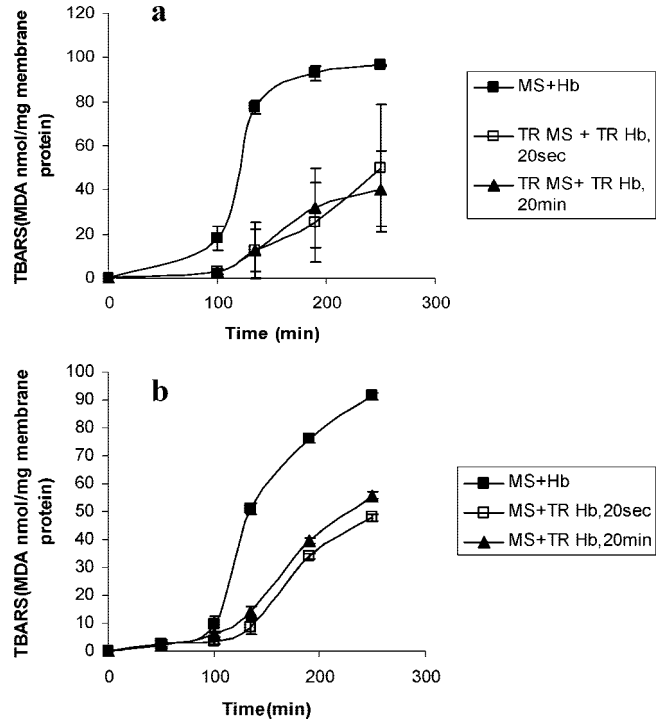

Figure 2. Effect of treating Hb at pH 3.0 for different times on its pro-oxidative activity (**a**) on treated microsomes and (**b**) on untreated microsomes ($n = 2$). MS, microsomal suspension. TR MS, MS exposed to pH 3.0 for 30 min and then readjusted to pH 6.8. Hb, hemoglobin. TR Hb, Hb exposed to pH 3.0 for 20 s or 20 min and then readjusted to pH 6.8. Treated or untreated Hb was added at a final concentration of 6 μM, and oxidation assays ran at pH 6.8.

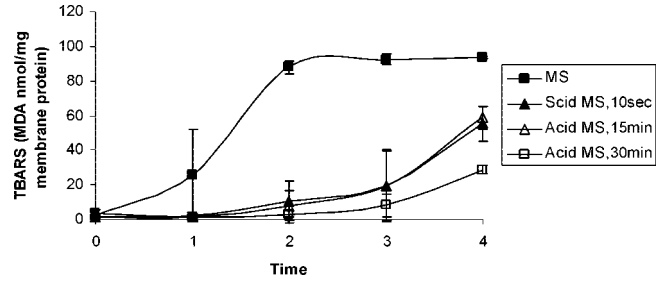

Figure 3. Effect of incubation time at pH 3 on the oxidation of acid-treated membrane suspensions with 6 μM untreated Hb ($n = 3$). MS, MS 0.7 mg/mL. Acid MS, 10 s, MS exposed to pH 3 for 10 s and readjusted to pH 6.8. Acid MS, 15 min, MS exposed to pH 3 for 15 min and readjusted to pH 6.8. Acid MS, 30 min, MS exposed to pH 3 for 30 min and readjusted to pH 6.8.

3.2. Effect of pH on NADH-Dependent and Fe-Ascorbate-Mediated Lipid Oxidation. Two low molecular weight iron systems that might play a role in initiating and promoting lipid

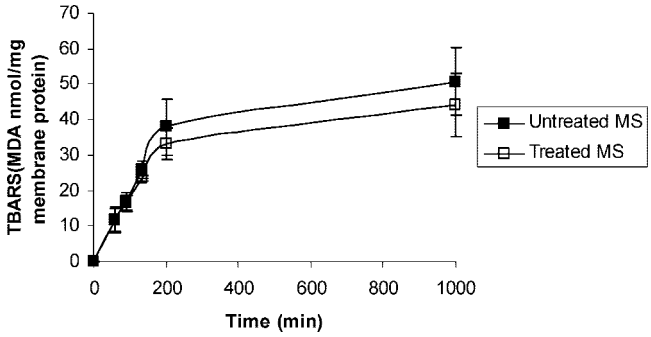

Figure 4. Nonenzymic oxidation of treated or untreated MSs ($n = 2$). Untreated MS, MS prepared at pH 6.8. Treated MS, MS prepared at pH 6.8; treatment at pH 3 for 1 h and then back to pH 6.8. Oxidation assays were run at pH 6.8; final concentrations of reactants were sodium ascorbate, 100 μM; ADP, 100 μM; and ferric chloride, 5 μM.

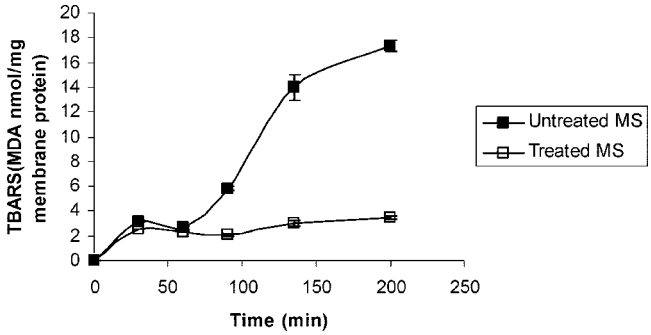

Figure 5. Effect of pH treatment on NADH-dependent microsomal lipid oxidation ($n = 2$). Untreated MS, MS prepared at pH 6.8. Treated MS, MS prepared at pH 6.8; treatment at pH 3 for 1 h and then back to pH 6.8. Oxidation assays were run at pH 6.8; the final concentrations of reactants were NADH, 100 μM; ADP, 100 μM; and ferric chloride, 5 μM.

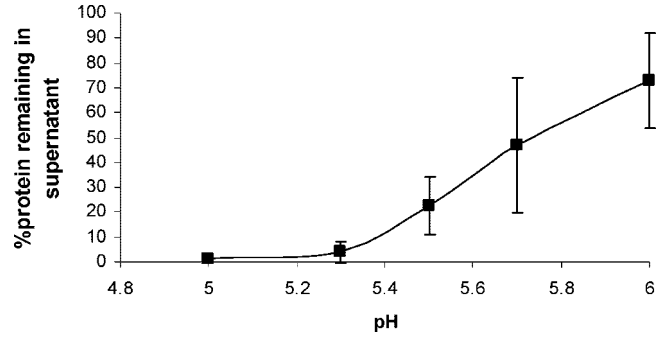

Figure 6. Sedimentation of isolated membrane as a function of pH in a 5 mM histidine–0.6 M KCl buffer. Isolated membranes were adjusted to pH values between 6 and 5 and centrifuged for 10 min at 2000*g*. Protein concentrations in the supernatants before and after centrifugation were determined.

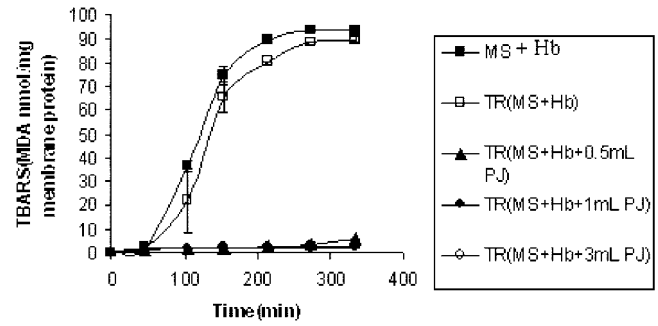

Figure 7. Oxidation of MSs treated at low pH together with Hb in the presence of different amounts of PJ. MS, MS and untreated Hb. TR (MS + Hb), MS and Hb exposed together at pH 3 for 15 min. TR (MS + Hb + 0.5 mL PJ), MS, untreated Hb, and 0.5 mL of PJ exposed together to pH 3 for 15 min. TR (MS + Hb + 1 mL PJ), MS, untreated Hb, and 1 mL of PJ exposed together at pH 3 for 15 min. TR (MS + Hb + 3 mL PJ), MS, untreated Hb, and 3 mL of PJ exposed together at pH 3 for 15 min. The membrane concentration was 0.7 mg/mL; Hb, 6 μM; final volume, 11 mL; and pH 6.8.

oxidation are those utilizing NADH (enzymic) or ascorbate (nonenzymic) to reduce ferric to ferrous iron. The treatment of MSs at low pH did not affect the rate of lipid oxidation when the iron–ascorbate system was used as a catalyst (**Figure 4**). Treatment of membranes at low pH greatly reduced the lipid oxidation produced by the enzymic system (NADH-ferric chloride). After an initial burst of oxidation, the production of TBARS plateaued at a low value when the microsomes had been exposed to pH 3 (**Figure 5**).

3.3. Effect of pH on Membrane Sedimentation. Liang and Hultin (*31*) showed that isolated cod muscle membranes could be readily sedimented at a low centrifugal force for 15 min at pH 5 or below but not at pH 6 or higher. No data in the literature were available for the pH range between 6 and 5. This is the range of interest of pH in the acid/alkali solubilization processes, since isoelectric precipitation of the muscle proteins is performed at around pH 5.5. Isolated membranes were prepared in histidine (pH 7.4). To study the precipitation behavior as a function of pH, samples were adjusted to pH 6, 5.8, 5.5, 5.3, and 5. After centrifugation at ~2000*g* for 10 min in a tabletop centrifuge, the supernatant was analyzed for protein content. The effect of pH on the ability to sediment membrane protein at 2000*g* for 10 min is shown in **Figure 6**. All of the samples had an exposure time of ~15 min (preparation + centrifugation time) at each pH. The results show that virtually all of the membranes (99%) sedimented at around pH 5.3 under these conditions.

3.4. Effect of PJ on Microsomal Lipid Oxidation. MSs and Hb in the presence or absence of PJ were exposed together to pH 3 for 15 min and then readjusted to pH 6.8. It was observed that even a 22-fold dilution of the PJ (0.5 mL of PJ in 11 mL of total volume) was enough to prevent Hb-induced microsomal lipid oxidation (**Figure 7**). We have reported that PJ at a six-fold dilution was able to prevent lipid oxidation of washed cod and recovered proteins from acid-solubilized cod muscle (*29*). PJ was able to retard lipid oxidation induced by the nonenzymic low molecular weight iron system (ascorbate–ferric chloride) even at a 22-fold dilution (**Figure 8**). From previous experiments, it was shown that acid treatment of membranes was able to prevent lipid oxidation induced by Hb (**Figure 3**) but not by the ascorbate–Fe system (**Figure 4**). PJ on the other hand was effective in both cases. PJ was also able to protect the MSs from NADH-dependent lipid oxidation, even when it was added at a 100-fold dilution (**Figure 9**).

3.5. Effect of Citric Acid and Calcium Chloride on Microsomal Lipid Oxidation. The effect of citric acid and calcium chloride on the oxidation of isolated membranes was tested in two different ways: (i) using citric acid and calcium chloride during the preparation of MSs and (ii) adding citric acid and calcium chloride directly to MSs previously prepared. Results of treating membranes by these two different approaches are illustrated in **Figure 10**. As controls, a standard microsomal preparation was used as well as a microsomal preparation that had been treated at pH 3. The catalyst in all cases was 6 μmol of native cod Hb. Treating the isolated membrane with 10 mM calcium chloride and 5 mM citric acid inhibited lipid oxidation

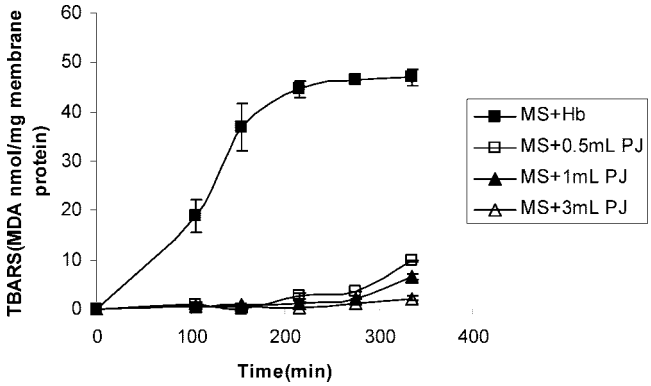

Figure 8. Microsomal lipid oxidation induced by the ascorbate-Fe^{3+} system and the effect of PJ ($n = 3$). MS, microsomal suspension; oxidation induced by the addition of the ascorbate-Fe^{3+} solution. MS + 0.5 mL PJ, MS and 0.5 mL of PJ (~22-fold dilution). MS + 1 mL PJ, MS and 1 mL of PJ (~11-fold dilution). MS + 3 mL PJ, MS and 3 mL of PJ (~3.5-fold dilution). The membrane concentration was 0.7 mg/mL, the final volume was 11 mL, and the pH was 6.8. The Asc–Fe system was used to initiate the reaction and consisted of 5 μM Fe^{3+}, 100 μM ascorbate, and 100 μM ADP.

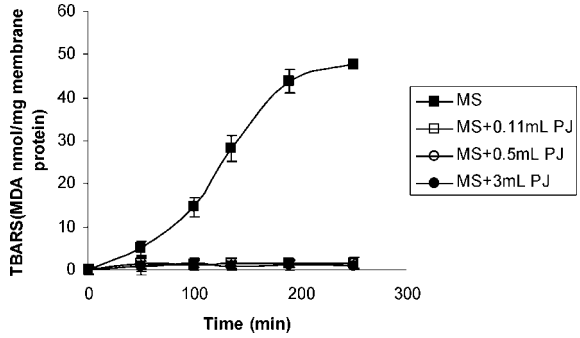

Figure 9. Effect of PJ on NADH-dependent lipid oxidation of microsomes ($n = 2$). MS, microsomal suspension. MS + 0.11 mL PJ, MS with 0.11 mL of added PJ. MS + 0.5 mL PJ, MS with 0.5 mL of added PJ. MS + 3 mL PJ, MS with 3 mL of added PJ. Lipid oxidation was induced by the addition of 10 μM $FeCl_3$, 100 μM ADP, and 100 μM NADH. The membrane concentration was 0.7 mg/mL, the final volume of assays was 11 mL, and the pH was 6.8.

quite markedly. A similar result was obtained if 5 mM citric acid and 10 mM calcium chloride were added to the homogenized muscle prior to isolation of the membranes. The addition of these components brought the pH of the homogenate mixture to about 5.4; this pH was raised to neutrality before the separation of the membranes took place by the usual technique. When treated with calcium chloride and citric acid at pH 5.4 or below, the yield of microsomes from the treated homogenate was lower than if it had not been treated with citric acid and calcium chloride (**Table 2**). We do not have any information at the present time as to how this lower recovery might have affected the nature of the microsomal preparation; that is, was there just a general reduction in yield or was there a selective reduction of membrane with special characteristics? This process gave almost the same inhibition as did the direct treatment of the isolated microsomes with the calcium chloride and citric acid. It was also similar to the inhibition observed when microsomes were prepared at pH 7.4 and exposed to pH 3 for 30 min before they were readjusted to pH 6.8.

Further experiments were done to test the effect of adding calcium chloride and citric acid on the changes that take place when Hb and the MS are exposed together at pH 3. A sample

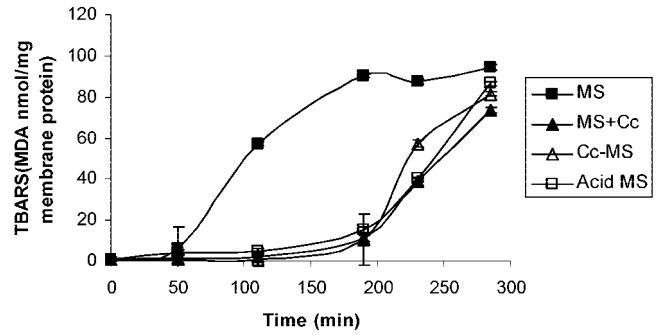

Figure 10. Effect of adding citric acid and calcium chloride to microsomes or used to prepare microsomes on Hb-mediated lipid oxidation. MS, MS in histidine buffer, pH 6.8. MS + Cc, citric acid and calcium chloride were added to the MS, and the pH was adjusted to 6.8. Cc-MS, MS prepared by addition of citric acid and calcium chloride (5 and 10 mM, respectively, final concentrations) in the mixture of minced cod and histidine buffer. Incubation took place at pH 5.45 for 30 min (i.e., pH 5.45 was the pH reached by the addition of citric acid and calcium chloride). The pH of the oxidation assay was 6.8. Acid MS, MS treated at pH 3 for 30 min and then readjusted to 6.8.

Table 2. Membrane Yields after Different Preparations ($n = 4$, ±SE)[a]

membrane preparation	membrane yield (mg membrane protein/mL)
MS at pH 7.2 w/o citric/calcium	5.47 ± 0.50 b
MS with citric/calcium (pH 7.2)	5.82 ± 0.39 b
MS with citric/calcium (pH 5.45)	4.49 ± 0.39 b
MS with citric/calcium (pH 5.2)	3.47 ± 0.54 c
MS with citric/calcium (pH 4.65)	1.46 ± 0.25 c

[a] Microsomes were prepared by adding citric acid and calcium chloride to the homogenate of minced cod, which was then adjusted to the pH values indicated by the addition of HCl. The homogenates were then incubated at their pH values for 30 min. Thereafter, the pH of all of the samples was adjusted to pH 6.8, and the microsomes were isolated as described in the Methods section. Means with the same letter are not significantly different ($p > 0.05$).

with untreated membranes and untreated Hb was compared to samples where both the membrane and the Hb were exposed to pH 3. In one case, the two components were exposed separately; in a second, they were exposed together; and in a third, they were exposed together in the presence of citric acid and calcium chloride. Results of these experiments showed that while treatment of the membrane suspension and the Hb together at low pH did cause a more rapid oxidation, the same samples to which the 5 mM citric acid and 10 mM calcium chloride were added had a rate of oxidation that was similar to the control of untreated microsomes and Hb; that is, the rate of oxidation in the presence of calcium chloride and citric acid was less than that in their absence when both the membrane and the heme protein were treated together at low pH for 30 min (**Figure 11**).

4. DISCUSSION

Pazos et al. (*23*) showed that low pH treatment of isolated cod microsomal membranes reduces Hb-mediated lipid oxidation. Isolated membranes assayed at pH 3.5 were more stable against lipid oxidation as compared to microsomes assayed at pH 6.8, particularly with respect to the length of the lag phase. It was suggested that structural and/or chemical changes induced by the low pH and aggregation of membranes in vitro due to the low pH might render microsomes less susceptible to Hb-mediated lipid oxidation. During acid solubilization, the muscle membranes and Hb are together exposed to the same treatment and conditions. When MSs (**Figure 1**) were exposed together

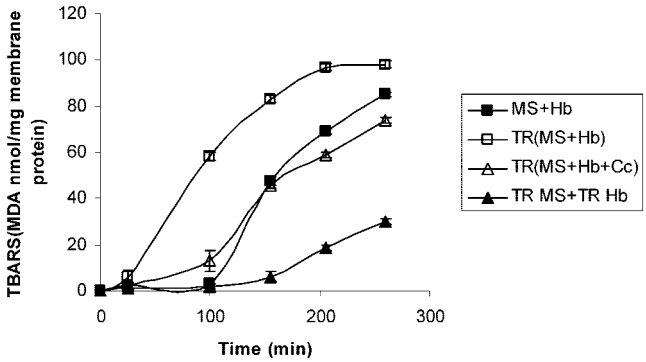

Figure 11. Effect of exposing MSs and Hb to pH 3 together in the presence of citric acid and calcium chloride. MS, MS and Hb. TR MS + TR Hb, MS treated at pH 3 for 30 min and Hb separately was treated at pH 3 for 20 s. TR (MS + Hb), MS and 6 μM Hb exposed together to pH 3 for 30 min before readjusting to pH 6.8. TR (MS + Hb + Cc), MS, 6 μM Hb and 5 mM citric acid/10 mM calcium chloride solution exposed together to pH 3 for 30 min before readjusting to pH 6.8. Oxidation assays were run at pH 6.8.

with Hb to low pH for 30 or 60 min, then oxidation was promoted as compared to untreated samples. Results in **Table 1** indicate that exposure times less than 30 min might or might not promote lipid oxidation. These observations suggest that time is an important factor and since the reactions that render the microsomes more susceptible to lipid oxidation are favored by a low pH, exposure time to low pH should be minimized. On the other hand, when microsomes and Hb were exposed to pH 3 separately and then mixed together at pH 6.8 to initiate the reaction, oxidation was significantly retarded (**Figure 2**). These observations are a clear indication that both the substrate and the pro-oxidant have to be present together at low pH to promote lipid oxidation.

To gain a better insight on the mechanism(s) involved, cod Hb and membranes were treated separately at low pH, and the effect of time was taken into consideration. An unexpected result was that brief exposure (20 s) of cod Hb to pH 3.0 (=treated Hb) led to partial loss of its pro-oxidative activity. Oxidation of treated and untreated microsomes using treated Hb as a catalyst was significantly decreased as compared to the oxidation mediated by untreated Hb (**Figure 2**). A longer exposure time of Hb to pH 3.0 (20 min) did not result in any further loss of pro-oxidative activity. These findings, however, do not give a satisfactory answer to the question of why simultaneous exposure of the muscle and Hb to low pH caused rapid oxidation.

The model of interaction between Hb and membranes that many authors adopt (*34–36*) is described by an initial reversible electrostatic interaction between the Hb and the membrane surface, followed by hydrophobic interactions and heme dissociation from the globin protein into the lipid phase of the membranes. When Hb is exposed to low pH, many structural and chemical changes take place, that is, dissociation of the heme, dissociation of the tetramer to monomers and dimers, autoxidation of the heme iron, and/or denaturation of the globin protein. When the Hb is treated alone at low pH, followed by readjustment to physiological pH, then some refolding of the protein takes place. Furthermore, the radicals produced during autoxidation of Hb can be quenched and autoreduced by the protein itself, resulting in free radical proteins that can cause intramolecular rearrangement of the protein (*37*). These changes in protein structure or loss of hemin on exposure to a low pH might cause the Hb to be a less effective pro-oxidant than the native proteins. On the other hand, when Hb is exposed to low pH in the presence of minced muscle, rapid oxidation is observed. This could be attributed to the lack of refolding ability due to competitive interactions with other unfolded proteins at that pH. Furthermore, low pH is expected to enhance the dissociation of heme from the globin moiety in the water phase (*38*), which is the rate-limiting step of the binding reaction of heme to liposomes (*34*).

In muscle tissues, Hb might not be the only pro-oxidant. NADH-dependent oxidation and iron-induced lipid oxidation have been shown to contribute significantly to oxidative deterioration of lipids in model systems (*39*, *13*). The oxidation of membrane lipids was not affected by low pH treatment when the oxidation catalyst was ascorbate and ferric iron (**Figure 4**). On the other hand, exposure of isolated membranes to low pH prevented the NADH-dependent (enzymic) system from catalyzing lipid oxidation (**Figure 5**), revealing one more possible mechanism through which low pH could protect the cod muscle from lipid oxidation.

There seemed to be a relation between the aggregation status of the membranes and their oxidative susceptibility. When the pH of the MSs was adjusted to 5.3 or lower, most of the membranes sedimented at low *g* force (**Figure 6**). It is likely that the net negative charge on the membranes is reduced to a value such that hydrophobic interactions become predominant, allowing nonspecific aggregation or fusion by means of the lipid bilayers. A number of studies have shown a pH-induced fusion of lamellar vesicles composed of amphipathic lipid molecules (*40–42*). In the case of biological membranes, there are proteins that have large surface areas on the surface of the membrane. Allowing the pH of the medium to approach the isoelectric point of the membrane proteins could cause nonspecific aggregation. It is interesting that the pH where most of the membranes sediment resides is similar to the range of the isoelectric point of myofibrillar proteins.

Another parameter that may play an important role in lipid oxidation is the physical state of the lipids. Raghavan and Hultin (*43*) showed that the amount of exogenous tocopherol incorporated into the membranes in competition with that taken up by triacylglycerols was higher when the triacylglycerols were solid than if the triacylglycerol was liquid, indicating that solid fat was not as receptive in absorbing the tocopherol as was the liquid oil. Light and Olson (*44*) indicated that the most important factor governing the rate and extent of CO–heme binding to liposomes was the physical state of the membrane. They showed that at 30 °C, the equilibrium association partition constant for CO–heme uptake by the liquid–crystalline phosphatidylcholine vesicles was 16-fold greater than that for liposomes in the gel state. It is possible that the pH treatments of the isolated membranes might alter their physical state and physicochemical properties, affecting the interaction of the membrane components with pro-oxidants, such as Hb. Low pH treatment results in a longer lag phase and lower oxidation rate. These observations are an indication that bound Hb might have restricted access to the substrate of lipid oxidation, that is, the phospholipids of the acid-treated membranes. Development of a gel phase, causing exclusion of the catalyst from the "solid" hydrocarbon interior, could be responsible for the restricted access of Hb. The resistance to Hb-mediated and/or NADH-dependent lipid oxidation that was exhibited by membranes treated at pH 3.0 (**Figures 4** and **6**) could be attributed to lipid phase changes that occur to the membranes when the pH is decreased to values below 5.3. Consistent with this view are the findings of Soyer and Hultin (*45*), who showed that the NADH-dependent

enzymic lipid oxidation system oxidized buried sulfhydryl groups in membranes more readily than did the nonenzymic iron–ascorbate system, which had a higher activity than the enzymic system for the oxidation of readily accessible surface groups. That the NADH–Fe–ADP system could not induce lipid oxidation of acid-treated membranes raises the possibility that this enzymic system did not have access to the interior of the treated membranes. Formation of the gel state would limit the functioning of the enzymic system towards the membrane phospholipids. Alternatively, the low pH could have destroyed the activity of the membrane NADH-driven Fe^{3+} reductase by denaturing the proteins.

It has been shown that aqueous extracts from some muscles inhibit Hb-mediated oxidation of cod muscle membrane lipids. Cod, mackerel, haddock, winter flounder, herring, and chicken aqueous extracts were able to significantly extend the lag phase of painty odor development in a washed cod model system or soybean phosphatidylcholine liposomes (*32*, *33*). In this study, when PJ was added to MSs at a 22-fold dilution, Hb-mediated lipid oxidation was greatly reduced (**Figure 7**). It is not known how the PJ protects the muscle from lipid oxidation. The antioxidative properties were attributed to the <1 kDa fraction of the PJ, which retained its properties after heating and centrifugation to remove coagulated proteins. A few low molecular weight compounds such as phosphates, spermine, trimethylamine oxide, and 2,3-diphosphoglycerate were tested but ruled out since they were unable to prevent oxidation to the same degree as the PJ (*32*). It was suggested that low molecular weight nucleotides and/or reducing agents might be involved in the inhibition. Undeland et al. (*32*) showed that PJ slightly accelerated the autoxidation rate of Hb in an aqueous test system. The suggested first step of the interaction of Hb with membranes is a reversible electrostatic binding to the membrane surface (*34*–*36*). PJ, which has a considerable ionic strength, 0.15–0.2 M, may decrease the pro-oxidative activity of Hb by hindering this binding. Thongraung et al. (*46*) showed that cod PJ improved the extractability of Hb added to minced cod and decreased the binding of soluble Hb to the sarcoplasmic reticulum.

PJ also retarded Fe–ascorbate-induced microsomal lipid oxidation as well as the NADH-dependent oxidation. Complete inhibition of the NADH-driven system was observed at a dilution of about 100-fold. It is really quite remarkable that the cellular soluble fraction has such strong antioxidative activity. Borhan et al. (*47*) showed that a dilution as high as 600-fold inhibited almost 80% of the lipid peroxidation activity of the sarcoplasmic reticulum of winter flounder muscle. Its effectiveness was only slightly reduced by heating.

Citric acid and calcium chloride added to microsomes in histidine buffer or used to prepare microsomes significantly protected the membranes from Hb-mediated lipid oxidation (**Figure 10**). Liang and Hultin (*48*) suggested that citric acid and calcium chloride might disconnect the linkage between membranes and cytoskeletal proteins. Citric acid might play a role as a competitor to the acidic phospholipids of membranes in binding to the basic amino acid residues of the cytoskeleton proteins that bind to the membranes (*49*, *50*). The membranes released from the cytoskeletal proteins could then aggregate due to either the final low pH or by cross-linking induced by Ca^{2+} (*31*), thus conferring protection from lipid oxidation. When membranes were prepared from muscle homogenate with the addition of citric acid and calcium chloride, the yield of membrane protein was always less as compared to the yield of membranes prepared without citric acid and calcium chloride (**Table 2**). Membranes could have been disconnected from cytoskeleton proteins and aggregated at the low pH caused by the addition of citric acid (pH ~5.2–5.45) in the homogenate or by cross-linking by calcium ions. It was shown that most of the isolated membranes in histidine buffer were sedimented at pH ~5.3 (**Figure 6**). The membranes may have remained aggregated after the muscle was neutralized and thus be removed by the first centrifugation step during the MS preparation. When membranes and Hb were together exposed to low pH in the presence of citric acid and calcium chloride, the oxidation rate was slowed down as compared to the samples exposed to the low pH without citric acid and calcium chloride. However, the presence of citric acid and calcium chloride did not improve the oxidative stability significantly as compared to the untreated MSs (**Figure 11**).

When fish muscle tissue is treated at low pH to solubilize proteins in preparation for protein isolation, lipid oxidation is accelerated. It had previously been shown that when isolated membranes were treated at low pH, there was an inhibition of Hb-catalyzed lipid oxidation. In this work, we have demonstrated that when the Hb is adjusted to a low pH in the presence of the membranes for a sufficiently long period of time, that is, 30 min or more, there is also an acceleration of lipid oxidation. Low pH makes the membranes of cod muscle less susceptible to oxidation, while it also reduces the pro-oxidative activity of cod Hb when these entities are treated at low pH separately. Myoglobin autoxidized more rapidly as compared to anodic Hb although the anodic Hb was a better catalyst for lipid oxidation in a washed cod muscle system at pH 6.3, suggesting that the rate of autoxidation was not the rate-limiting step in the oxidation process (*51*). In a further study, Grunwald and Richards (*52*) observed that myoglobin variants with low heme affinity were strong pro-oxidants, whereas a variant with high affinity for hemin was a relatively poor pro-oxidant, again suggesting that it was the release of the heme pigment that controlled the rate of lipid oxidation. They further showed that a myoglobin variant that was sensitive to heme degradation and the release of iron was also a poor pro-oxidant. These results suggest that the loss of heme from Hb (presumably following dissociation of the tetrameric protein to monomers or dimers) followed by interaction with the lipid substrate are responsible for lipid oxidation. The high hydrophobicity of the heme moiety is consistent with this explanation. This may possibly indicate that the treatment of heme protein at low pH in the presence of the membrane allows the direct release and interaction of the released hemin with the membrane lipids. When the membrane is exposed separately, it may undergo chemical and physical changes, which either lower the interaction of the Hb with membranes or make the lipid go into a structural state by which the sensitive polyunsaturated fatty acids are protected by restricted access to the released heme. Likewise, if the Hb is exposed to low pH, any released heme may undergo autoxidation to a less active form of the heme compound, or the released heme may interact nonspecifically with hydrophobic areas on the surface of any proteins available, for example, the Hbs, and not have access to the lipids.

ABBREVIATIONS USED

Hb, hemoglobin; treated HB, hemoglobin exposed to pH 3 and readjusted to ph 6.8; MS, microsomal suspension; treated MS, microsmal suspension exposed to pH 3 and readjusted to pH 6.8; TBARS, thiobarbituric acid reactive substances.

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