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Hemoglobin-Mediated Oxidation of Washed Minced Cod Muscle Phospholipids: Effect of pH and Hemoglobin Source

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Lipid pro-oxidative properties and deoxygenation/autoxidation patterns of hemoglobins from nonmigratory white-fleshed fish (winter flounder and Atlantic pollock) and migratory dark-fleshed fish (Atlantic mackerel and menhaden) were compared during ice storage at pH 7.2 and 6. A washed cod mince model system and a buffer model system were used for studying lipid changes and hemoglobin changes, respectively. TBARS and painty odor were followed as markers for lipid oxidation. At pH 6, all four hemoglobins were highly and equally active as pro-oxidants. At pH 7.2, pro-oxidation by all hemoglobins except that from pollock was slowed down, and activity ranked as pollock > mackerel > menhaden > flounder. The higher catalytic activities of the hemoglobins at pH 6 than at pH 7.2 corresponded with higher formation of deoxyhemoglobin and methemoglobin. Pollock had the most extensive formation of deoxy- and methemoglobin at both pH values, which could explain its high catalytic activity. The pro-oxidative differences among the other hemoglobins at pH 7.2 did not correlate with deoxygenation and autoxidation reactions. This indicates involvement of other structural differences between the hemoglobins such as differences in the heme-crevice volume. It is suggested that a biological reason for the species differences was their adaptations to different depths/water temperatures.

KEYWORDS: Hemoglobin; pH effects on hemoglobin; oxidation; cod; pollock; flounder; menhaden; mackerel

INTRODUCTION

Lipid oxidation is one of the major reactions behind quality losses in fish muscle during storage, with many muscle components having the potential to participate and/or act as catalysts. Currently, considerable attention is given to hemoglobin as a potent lipid oxidation catalyst in muscle. Hemoglobin, the function of which is to transport O_2, H^+, and CO_2, is the main protein in blood, where it is highly concentrated in the erythrocytes at above ∼300 mg/mL (*1*, *2*). Post-mortem, there are many opportunities for hemoglobin to become mixed with lipid substrates and effectors. Blood capillaries can burst naturally but also during mechanical treatments such as filleting and mincing. The liberated erythrocytes can become disrupted, for example, by mechanical treatment, after washing with water or hypotonic salt solutions, or after oxidative destruction of the erythrocyte cell membrane. Richards and co-workers (*3*, *4*) found that bleeding of mackerel and trout prior to mincing of the fillet significantly reduced the rate of rancidity development after storage of the mince on ice. It was found that hemoglobin accounted for all of the pro-oxidative potential of the blood that was removed during the bleeding (*4*). In a washed cod mince model system, it was found that trout hemoglobin was a more potent catalyst of oxidation than were equimolar levels of Fe^{3+}-ADP reduced by ascorbate (*5*) or by endogenous microsomal enzymes, NADH and histidine (*6*).

The pro-oxidative activity of fish hemoglobin in vitro has been found to increase following the small reductions in pH within the span that naturally occur during post-mortem storage of the fish (e.g., from pH 7 to 6.2) (*7*). When pro-oxidative activity of trout hemoglobin toward the phospholipids of washed cod mince was tested at pH 6, 7.2, and 7.6, the lag phase before the onset of oxidation was 0, 2, and 12 days, respectively (*6*−*8*). Lowering the pH from neutrality leads to deoxygenation of hemoglobin, called the Bohr effect or the Root effect (an exaggerated acid-shifted Bohr effect) (*9*). Richards and Hultin (*7*) proposed that increased deoxygenation as pH is lowered could play a role in the increased pro-oxidative activity of hemoglobin. The protein subunit association is shifted upon deoxygenation, resulting in a more exposed state of the heme

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group (*2*−*10*). This would facilitate decomposition of lipid hydroperoxides into lipid radicals and volatiles (*11*). Hemoglobin deoxygenation is also thought to trigger hemoglobin autoxidation, which can lead to production of active oxygen species (*12*, *13*), ferryl-Hb radicals (*14*), and heme dissociation (*15*).

Fish hemoglobins operate under much greater variations in their environmental conditions than hemoglobins from air-breathing vertebrates (*9*). Dependent on fish species and developmental stage of the fish, there are sometimes more than one electrophoretically distinct form of hemoglobin, each with its own response to pH and its own catalytic properties (*16*). It is hypothesized that migratory fast-swimming pelagic species require hemoglobin that can rapidly liberate oxygen; thus, they have a less stable tertiary structure. This may possibly make them more active in catalyzing lipid oxidation than hemoglobins from nonmigratory species. Richards and Hultin (*17*) recently found that hemoglobin from mackerel, a migratory pelagic fish, was more pro-oxidative than hemoglobin from trout, a less active species. A better understanding of such differences could aid in the development of species-specific antioxidative strategies to apply during the processing of fish.

As only a handful of fish hemoglobins have been the subject of study for their potential to catalyze lipid oxidation, the aim of this study was to evaluate the pro-oxidative effect of hemoglobins from two migratory pelagic species (Atlantic mackerel and menhaden) and two nonmigratory whitefish species (Atlantic pollock and winter flounder). A second aim of our study was to investigate how the pro-oxidative activity of these hemoglobins would be affected by pH variations within the range commonly seen in post-mortem fish, pH 7.2−6. To be able to explain pH- and/or species related differences in the pro-oxidative activity of the hemoglobins, we also measured storage-induced hemoglobin deoxygentation and autoxidation in a buffer-based aqueous model system.

MATERIALS AND METHODS

Bleeding of Fish and Preparation of Hemolysate. Menhaden (*Brevoortia tyrannus)* (size ∼5 cm), mackerel (*Scomber scombrus*) (size ∼40 cm), winter flounder (*Pleuronectes americanus*) (size ∼30 cm), and Atlantic pollock (*Pollachius virens*) (size ∼20 cm) were anesthetized and bled as described by Rowley (*18*). Hemolysates were prepared from the whole blood according to the method of Fyhn et al. (*19*) by washing the red blood cells four times in 1 mM Tris (pH 8) containing 290 mM NaCl and then lysing them in 1 mM Tris (pH 8). To quantify the hemoglobin (Hb) levels in the hemolysates, the method of Brown (*20*) was adapted as described by Richards and Hultin (*7*). The purity of the hemolysate was analyzed by SDS-PAGE and densitometry using previously described procedures (*21*). Low molecular weight compounds were removed from the flounder hemolysate by dialysis of 5 mL of hemolysate against 2 L of 1 mM Tris (pH 8) using a 3.5 kDa membrane for 24 h at 4 °C. The buffer was changed three times.

Washed Cod Muscle Model System. Whole cod (*Gadus morhua*) was obtained in rigor from local fishermen in Gloucester, MA, and filleted on arrival to the laboratory. The white muscle was removed manually and ground with an Ultra power grinder (model KS M90, KitchenAid Inc., St. Joseph, MI). Washing was done according to the method of Richards and Hultin (*7*) with the modification that the last wash in 50 mM phosphate buffer was done either at pH 6 or at pH 7.2 instead of at pH 6.7. The final washed cod mince was frozen at −80 °C.

Preparation of Oxidation Model System. The washed mince was thawed in a sealed plastic bag under running cold water. Moisture was determined with a moisture balance (CSC Scientific Co. Inc.), and the pH was measured using a Ross Sure-Flow pH-electrode (model 8165 combination epoxy electrode, Orion, Beverly, MA). The pH of washed cod mince samples was measured after manually stirring 1 part mince with 9 parts Milli-Q water. The moisture of the pH 6 model system (80%) was adjusted to that of the pH 7.2 model system (90%) with 50 mM phosphate buffer (pH 6). In the model system used to study dialyzed and nondialyzed flounder hemolysates, the washed cod mince model had a pH of 7 and 88% moisture.

To prepare each sample for lipid oxidation studies, 30 g of the washed cod model was weighed into a 50 mL plastic beaker. Two hundred parts per million of streptomycin was added to prevent bacterial growth, and lipid oxidation was started by adding fish hemolysate to a final hemoglobin concentration of 6 μmol/kg of washed cod mince. Antimicrobial agent and pro-oxidants were stirred into the cod model by hand (2 min, ∼160 turns/min) using a stainless steel spatula. In controls, the hemolysate was replaced by distilled water. The samples were flattened out in the bottom of 225 mL screw-capped Erlenmeyer flasks (bottom diameter = 75 mm) using an L-shaped stainless steel spatula. The sample thickness became ∼6 mm. The capped sample bottles were stored on ice. Samples containing mackerel hemolysate at pH 6 were prepared in triplicate, the other samples as singles. See further details on replication under Experimental Setup and Analysis of Data.

Analysis for Lipid Oxidation Products (TBARS). At regular intervals, 1 g sample "plugs" were taken from the samples for TBARS analyses. The plugs were removed using a plastic cylinder (diameter = 1 cm) and, thus, had constant surface-to-volume ratios. The samples were stored in aluminum foil in a sealed plastic bag at −80 °C until the day of analyses. TBARS were analyzed after extraction of the muscle with trichloroacetic acid (*22*) and are expressed as micromoles of malonaldehyde (MDA) per kilogram of tissue.

Total Lipid Analysis. Total lipids in washed cod mince were analyzed according to the method of Lee et al. (*23*) using chloroform and methanol (1:1) as the extraction solvent.

Sensory Analysis. Once to twice daily, three to four trained panelists (*3*) sniffed the headspace above the samples by uncapping the 225 mL screw-capped Erlenmeyer flasks. The sample set, which consisted of 12 bottles, was split into two sets of six samples each. The panelists were rested for 45 min between evaluations of each set. Panelists concentrated on detecting painty odors using a scale of 0−10, with 10 being the strongest. The panelists used the same sample sets for sensory evaluation. The sample flasks were therefore held on ice for 1 h between panelists so that the volatiles could reach a new equilibrium between the cod mince model and the headspace above. Reference samples were prepared according to the method of Richards et al. (*3*). The lag phase for painty odor development was defined as time elapsing until an intensity of 1 was reached.

Hemoglobin Oxygenation. Hemolysate was added to 50 mM sodium phosphate buffers at pH 6 and 7.2 to give a solution of 6 μM hemoglobin. The hemolysate solutions were stored in test tubes at 2 °C and scanned from 500 to 630 nm against a buffer blank using a double-beam Hitachi U-3110 spectrophotometer (Hitachi Instruments, Inc., San Jose, CA). Samples were scanned at the same time points as oxidation was assessed. The amount of oxyhemoglobin in the different hemolysates was estimated as the peak absorbance at 575 nm minus the "valley" absorbance at 560 nm (*24*). **Figure 1** illustrates a typical scan of a flounder hemolysate solution at pH 7.2 before and after 15 and 120 h of storage on ice. The "peak" at 575 nm and the "valley" at 560 nm are clearly seen. Because the oxygenation estimation is performed in the same wavelength range as autoxidation is calculated (see below), the oxygenation data might be influenced by concurrent autoxidation. Therefore, data on oxyhemoglobin are reported at only time 0. We have also given the time period that elapsed until oxyhemoglobin could no longer be detected.

Hemoglobin Autoxidation. Autoxidation of hemoglobin was followed at 2 °C for 5 days in the hemolysate solutions (∼6 μM hemoglobin) as prepared above (in 50 mM sodium phosphate buffer at pH 6 and 7.2). To follow hemoglobin autoxidation over time, hemolysate solutions were measured at 575 nm (**Figure 1**) against a buffer blank at the same time points as oxidation was assessed. The relative rate and extent of hemoglobin autoxidation was determined from the formula by Tajima and Shikama (*13*), assuming that loss of reduced oxyhemoglobin was equal to formation of methemoglobin:

$$\text{autoxidation rate} = \ln(\text{HbO}_2)_t/(\text{HbO}_2)_0$$

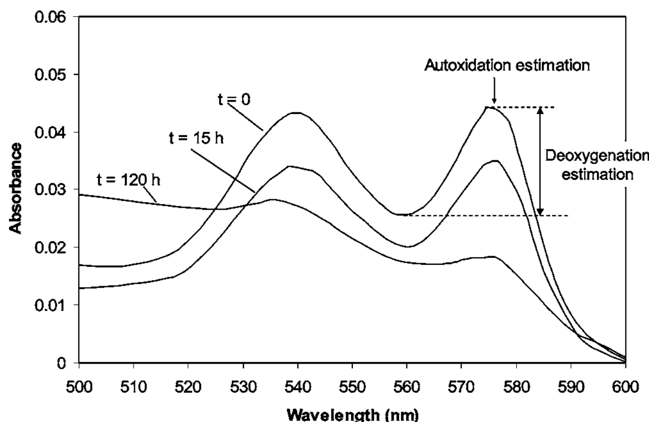

Figure 1. Typical scan of a solution consisting of flounder hemolysate (6 μM hemoglobin) in 50 mM phosphate buffer (pH 7.2) between 500 and 600 nm after 0, 15, and 120 h on ice. The figure shows where in the scan the changes that were used for estimation of deoxygenation and autoxidation took place—at 560 and 575 nm.

$(HbO_2)_t$ is the fraction of reduced oxyhemoglobin at time t, and $(HbO_2)_0$ is the fraction of reduced hemoglobin at the start of the experiment. $(HbO_2)_t$ is determined by the absorbance ratio of

$$(\mathrm{HbO_2})_t = \left\{\frac{(A_{575,t} - A_{575,\infty})}{(A_{575,0} - A_{575,\infty})}\right\}$$

where $A_{575,t}$ and $A_{575,0}$ are the absorbances at 575 nm at time t and at the start of the experiment, respectively. This formula assumes that when no further change has occurred in absorbance at 575 nm (i.e., $A_{575,\infty}$), the reduced oxyhemoglobin has been fully oxidized. In most cases, hemoglobin experienced biphasic autoxidation, why the k value (k_{obs}) describing the entire reaction ($HbO_2 \rightarrow$ met-Hb + O_2^-) was broken down into two rate constants, one representing the initial fast autoxidation (k_f) and one representing the second slow autoxidation (k_s) (*6*, *8*).

Experimental Setup and Analysis of Data. The use of sensory analysis limited the possible number of washed cod mince samples within each storage trial to 12. This sample number yielded two individual sensory sets of six samples each. These sets were smelled once or twice daily (at each smelling occasion, $a = 1$). At each time point and for each sample, we report an average data from all of the panelists. To still get an estimate of the sample-to-sample variation, we chose to make three replicates ($n = 3$) of the sample that was expected to change the most over time (washed cod mince at pH 6 with mackerel hemoglobin). Standard deviations (SD) from this triplicate were calculated using (Excel 2000, Microsoft Corp., Seattle, WA) and are shown as error bars in **Figures 2a** and **3a**. To confirm our general conclusions, the entire storage trial was repeated using a different batch of washed cod. TBARS analyses supported the sensory analyses during one of these storage trials. For samples with added pollock hemoglobin, a third replication was used. The reason for not merging sensory data from the two individual experiments in which different batches of raw material had been used was that this would hide valuable information on sample differences in batch-to-batch differences.

For TBARS analyses, TCA extracts were done on two "tissue plugs" from each sample bottle, and TBARS were analyzed twice on each of these two extracts ($a = 2$). An average of the analytical data was used to calculate the sample average shown in figures. Total lipids were extracted from three washed cod mince samples, and results are given as average ± standard deviation (SD). The hemolysate/buffer solutions were prepared as single samples but analyzed twice ($a = 2$) for absorbances at 575 and 560 nm. On the basis of these two values, average values on oxygenation/autoxidation status are given.

RESULTS

Development of Hemoglobin-Mediated Lipid Oxidation. On addition to the washed cod mince (5.3 ± 0.2% lipids on a dry weight basis), hemoglobin from all four species induced rapid development of painty odor at pH 6 (**Figure 2a**). Pollock hemoglobin oxidized the lipids of the washed cod mince at the fastest rate and flounder hemoglobin at the slowest rate. Oxidation induced by pollock hemoglobin exhibited no lag phase, whereas the other three hemoglobins gave rise to ~0.2 day lag phases. After 1.7 days, samples containing pollock, mackerel, or menhaden hemoglobins had reached similar levels of painty odor intensity and further changes were very small. Painty odor in the sample containing flounder hemoglobin had not reached a maximum level at 4 days. At pH 7.2, pollock hemoglobin induced painty odor in the washed mince at the same fast rate as at pH 6 (**Figure 2b**). However, in samples containing hemoglobin from the other species, painty odor was not detected until 1 day (mackerel) to 2.7 days (menhaden and flounder) after their addition. Whereas the paintiness induced

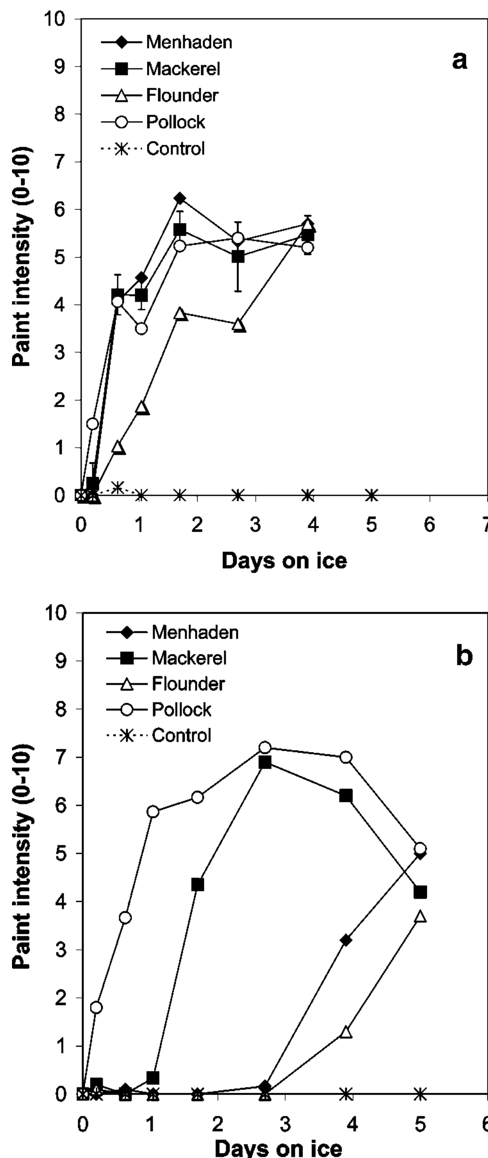

Figure 2. Development of painty odor in washed cod mince at (**a**) pH 6 and (**b**) pH 7.2 with menhaden, mackerel, flounder, and pollock hemolysates added as catalysts. Hemolysates were added to give 6 μmol of hemoglobin/kg of washed mince, and the samples were kept on ice. Final moisture in the system was 90%. Error bars in (**a**) show standard deviations for three replicate samples containing mackerel hemolysate. The other samples were stored as singles. The entire experiment was repeated twice.

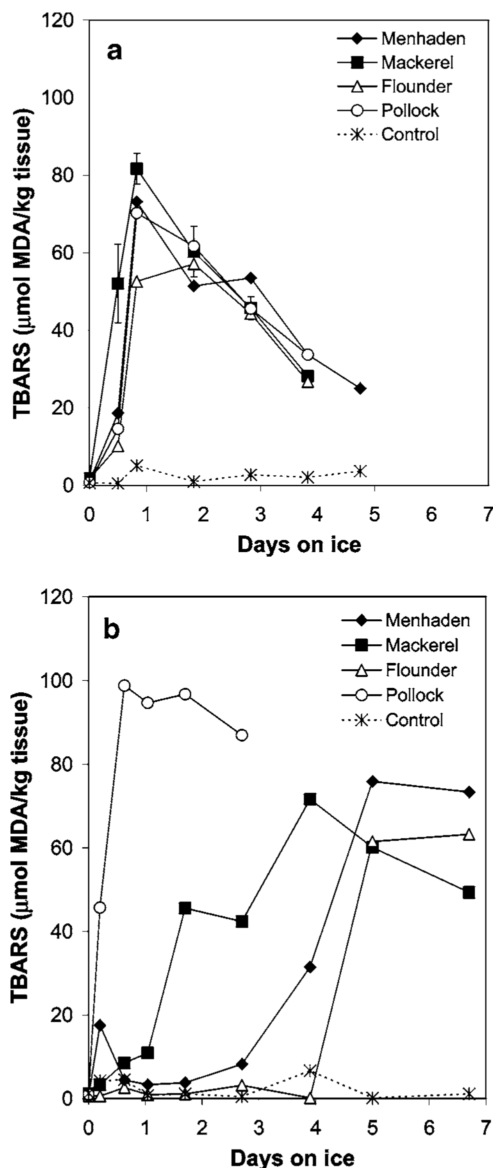

Figure 3. Development of TBARS in washed cod mince at (**a**) pH 6 and (**b**) pH 7.2 with menhaden, mackerel, flounder, and pollock hemoglobin added as catalysts. Hemolysates were added to give 6 μmol of hemoglobin/kg of washed mince, and the samples were kept on ice. Final moisture in the system was 90%. Error bars in (**a**) show standard deviations for three replicate samples containing mackerel hemolysate. The other samples were stored as singles. TBARS were analyzed in duplicate on each sample with average values shown or used for further calculations.

by pollock and mackerel hemoglobins started to decline after 2.7 days, the samples containing menhaden and flounder hemoglobins did not reach a maximum value within the studied period. During replication of the experiment, the same trends for painty odor development were seen as a result of varying the pH or the source of hemoglobin. Oxidation lag phases were, however, ~0.5−1.5 days longer than in the trial shown in **Figure 2**. In the extra experiment using only pollock hemoglobin, pH still had no effect on its lipid pro-oxidative capacities, although a 0.8 days lag phase was seen at both pH values.

The same samples that were subjected to sensory testing were also analyzed for thiobarbituric acid reactive substances (TBARS). In agreement with the sensory studies, TBARS values developed very quickly at pH 6, with no lag phases observed in the presence of any of the hemoglobins (**Figure 3a**). Mackerel hemoglobin catalyzed TBARS formation somewhat more quickly than the other hemoglobins. Between 1 and 1.7 days, all samples reached maximum TBARS values, after which the values dropped. The maximum thus occurred 1−1.5 days earlier than for painty odor, which is in agreement with earlier studies (*6*, *8*). At pH 7.2, TBARS data agreed well with sensory results in that oxidation was significantly slowed down for all samples except for that containing pollock hemoglobin (**Figure 3b**). The lag phases as measured by TBARS were 1, 2.7, and 3.9 days for catalysis due to mackerel, menhaden, and flounder hemoglobin, respectively. There was also a corresponding delay in the point where maximum TBARS values were observed. The TBARS values given by pollock hemoglobin were 30% lower at pH 7.2 (~70 μmol of MDA/kg) than at pH 6 (~100 μmol of MDA/kg). In both sensory and TBARS studies, the controls with no added hemoglobin did not change noticeably on storage.

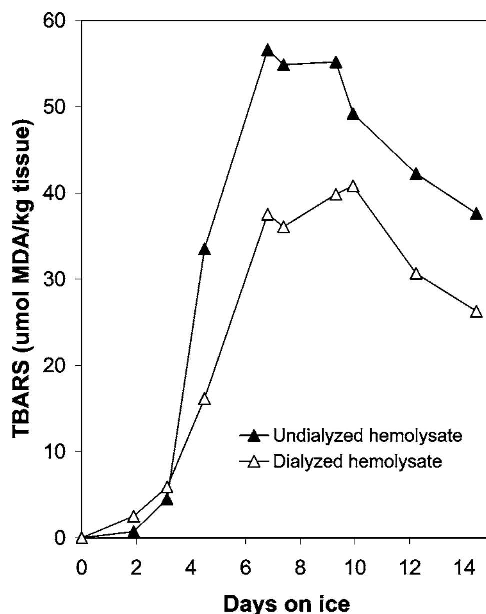

Figure 4. Development of TBARS in washed cod mince at pH 7 with nondialyzed and dialyzed flounder hemolysates added as catalysts. Hemolysates were added to give 6 μmol of hemoglobin/kg of washed mince, and the samples were kept on ice. Final moisture in the system was 88%. TBARS were analyzed in duplicate on each sample with average values shown.

Because flounder hemoglobin catalyzed lipid oxidation less than the hemoglobins from other species at pH 7.2, it was evaluated whether this was due to the presence of low molecular weight compounds with tentative antioxidative effects in the flounder hemolysate. **Figure 4** shows that the lag phase in oxidation due to the addition of dialyzed and undialyzed flounder hemolysates were similar but that the maximum TBARS level from the dialyzed sample was reduced by almost 30% (from 60 to 43 μmol of MDA/kg).

Deoxygenation and Autoxidation of Hemoglobin. An aqueous model system was used to estimate initial oxygenation and the autoxidation over time of the four hemoglobins at pH 6 and 7.2 (**Tables 1** and **2**; **Figure 5**). At time 0, all four hemoglobins were less oxygenated at pH 6 compared to at pH 7.2 (**Table 1**), which indicates that deoxygenation took place immediately upon mixing of the hemolysate into the pH 6 buffer. At pH 6, menhaden hemoglobin was initially more oxygenated than the other three hemoglobins, and it was possible to detect the oxy form for a longer period of time (2.1 days compared to <0.4 days). The other hemoglobins had similar oxygenation values at time 0, but already after 0.1 days, it was no longer possible to detect pollock oxyhemoglobin.

Table 1. Levels of Oxyhemoglobin Expressed as $A_{575} - A_{560}$[a] in Hemolysates from Pollock, Flounder, Menhaden, and Mackerel at pH 6 and 7.2

hemoglobin source	pH 6 $A_{575} - A_{560}$	days until no oxyhemoglobin detected	pH 7.2 $A_{575} - A_{560}$	days until no oxyhemoglobin detected
pollock	0.0037	0.1	0.0136	>5
flounder	0.0041	0.4	0.0186	>5
menhaden	0.0079	2.1	0.0196	1.5
mackerel	0.0038	0.3	0.204	>5

[a] Absorbances were measured in duplicate on each sample, with average values used for calculations of relative oxygenation.

Table 2. Rate Constants (k) for the "Fast" (k_f)[a] and "Slow" (k_s)[a] Phases of the Hemoglobin Autoxidation Reaction at pH 6 and pH 7.2 (See **Figure 5**)

hemoglobin source	pH 6		pH 7.2	
	k_f (h^{-1})	k_s (h^{-1})	k_f (h^{-1})	k_s (h^{-1})
pollock	0.07	0.0012	0.032	0.0056
flounder	0.065	0.0024	0.0082	b
menhaden	0.065	0.0055	0.0092	b
mackerel	0.045	0.0032	0.011	b

[a] At pH 6, the transition between the "fast" and "slow" periods was at 17 h for pollock, 25 h for mackerel, and 41 h for menhaden and flounder. At pH 7.2, the transition for pollock was at 41 h. [b] The autoxidation reaction followed a linear reaction and was therefore not broken down into two phases.

Changes in hemoglobin autoxidation at the two pH values are shown in **Figure 5**. At pH 6, both fast and slow rates of hemoglobin autoxidation could be identified. At pH 7.2, fast and slow rates were only evident for autoxidation of pollock hemoglobin, whereas the other hemoglobins exhibited a linear increase in autoxidation. At both pH values, the rate constants were calculated for the initial period of fast autoxidation (k_f) and for the second period of slower autoxidation (k_s) (**Table 2**). During the fast phase at pH 6, hemoglobin from pollock oxidized at the highest rate ($k_f = 0.07$ h^{-1}), followed by flounder and menhaden hemoglobins ($k_f = 0.065$ h^{-1}) and then mackerel hemoglobin ($k_f = 0.045$ h^{-1}). During the second phase of slow autoxidation (k_s) at pH 6, the rates of the hemoglobins followed the order menhaden > mackerel > flounder > pollock. At pH 7.2, autoxidation proceeded considerably more slowly than at pH 6 (**Figure 5b**; **Table 2**). On the basis of the changes during the fast period, pollock hemoglobin still exhibited the highest rate of oxidation ($k_f = 0.032$ h^{-1}) with hemoglobins from mackerel, menhaden, and flounder (in this order) showing a slower but similar rate of oxidation.

Correlations between Lipid Oxidation and Hemoglobin Oxidation. At pH 6 and 7.2, autoxidation of the four hemoglobins was overall highly correlated with TBARS development. r values (Pearson's correlation coefficient) varied between 0.80 and 1.0. Exceptions were the correlation given by flounder hemoglobin at pH 7.2 ($r = 0.67$) and by menhaden hemoglobin at pH 6 ($r = 0.73$). The correlation between autoxidation of the four hemoglobins and painty odor development was between $r = 0.80$ and $r = 0.98$ at both pH values.

DISCUSSION

The use of washed minced cod muscle as a model system provides a matrix that has the structure of muscle, that is, with intact myofibrillar proteins and membranes but virtually free of endogenous triacylglycerols and pro- and antioxidants.

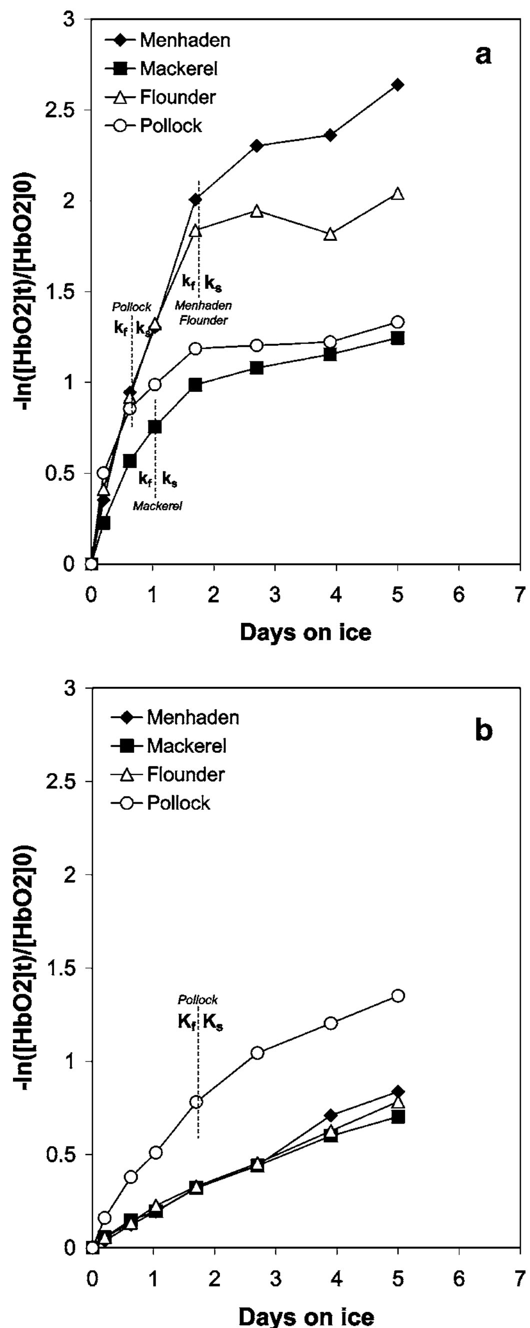

Figure 5. Autoxidation rate of menhaden, mackerel, flounder, and pollock hemoglobin at (**a**) pH 6 and (**b**) pH 7.2. The hemolysates were diluted to 6 μM in 50 mM sodium phosphate and stored on ice. In cases where two rate constants (k_f and k_s) were calculated to describe the autoxidation rate, the transition point between these is shown by a dashed line. Absorbances were measured in duplicate on each sample, with average values used for calculations of met-hemoglobin.

Controlled physiological levels of oxidation catalysts can then be added and studied in relation to lipid oxidation under various conditions of pH.

Painty odor and TBARS data showed that added fish hemoglobins effectively catalyzed oxidation of membrane lipids in washed minced cod muscle (**Figures 2** and **3**). The detected decline in oxidation products, particularly TBARS, could indicate that these oxidation products reacted further with compounds in the washed cod mince model system. Likely reactions of aldehydic secondary oxidation products in muscle are those with free amino groups, for example, in membrane

lipids, proteins, and peptides to form Schiff's bases (*25*). Reactions with active methylene compounds such as in histidine to form Michael addition products are also possible (*26*, *27*). The repeated "uncapping" of sample flasks during sensory analysis sessions could be an additional reason for loss in volatiles contributing to painty odor.

Effect of pH on Lipid Oxidation and Hemoglobin Changes. In samples containing mackerel, menhaden, and flounder hemoglobin, lipid oxidation was accelerated at pH 6 as compared to pH 7.2 (**Figures 2** and **3**). This was in agreement with previous findings (*4*, *6*) and may be connected to the reduction in relative oxygenation (Bohr or Root effect) and/or the increased rates with which these hemoglobins oxidized at pH 6 compared to pH 7.2 (**Tables 1** and **2**; **Figure 5**). It has been suggested (*28*) that deoxyhemoglobin acts as a stronger oxidation catalyst than oxyhemoglobin because it has a heme-crevice that is more accessible (*10*). The iron atom of the porphyrin group inside the crevice is also kicked out of the plane when deoxygenation occurs (*2*). This allows the iron to more easily interact with lipid hydroperoxides and, for example, hydrogen peroxide, creating more free radicals to facilitate both hemoglobin autoxidation and lipid oxidation.

Heme autoxidation can influence lipid oxidation by giving rise to oxygen radicals ($O_2^{\bullet -}$, $^{\bullet}OH$) (*12*), protein radicals (e.g., hypervalent ferryl-hemoglobin ($Fe^{4+}{=}O$)),[14] and lipid radicals ($LOO^{\bullet}$ and $LO^{\bullet}$) (*29*). In addition, it was shown (*15*) that methemoglobin is ~60 times more likely to release its heme group as compared to oxy- and deoxyhemoglobin. Due to its low polarity, free heme is expected to dissolve into the hydrophobic interior of membranes. When a critical level of peroxides is formed, iron can also be released from hemin and act as a low molecular weight initiator of lipid oxidation (*30*). Acid can also catalyze hemoglobin dissociation. Human hemoglobin dissociated into dimers 10 times more quickly at pH 6.2 than at pH 7.5 (*31*). It was reported that dimers undergo autoxidation 16 times faster than tetramers (*32*).

The lack of differentiation in lipid oxidation between samples at pH 6 could indicate that the pH reduction affected the washed cod mince model system itself and/or the contact between the membrane phospholipids and the hemoglobin molecules. During acidifications of fish muscle homogenates in our laboratory (unpublished data), increased binding (coprecipitation) of sarcoplasmic proteins with membranes was observed. Membrane changes could be caused, for example, by protonation of the phospholipid headgroup (*33*) or by lipolysis.

Lipid oxidation catalyzed by pollock hemoglobin was equally intense at both of the studied pH values, with virtually no lag phases seen at either pH 6 or 7.2. On the basis of the discussion above, this finding was possibly connected to the relatively low degree of oxygenation and the high autoxidation rate of pollock hemoglobin at pH 7.2 (**Figure 5**; **Tables 1** and **2**). Possibly, the relative amounts of deoxy- and/or methemoglobin initially present in the pollock hemolysate at pH 7.2 were already above the levels needed for immediate initiation of oxidation of the cod muscle membrane lipids. This indicates that pollock hemoglobin has its Bohr effect located well above pH 7.2. This appeared to be different from the other hemoglobins.

Species Differences among the Tested Hemoglobins. At pH 6, there were only small differences in the abilities of the four hemoglobins to catalyze painty odor (**Figure 2a**) and TBARS development (**Figure 3a**). Most obvious was the lower efficiency of flounder hemoglobin to catalyze painty odor development. However, during early storage, mackerel hemoglobin also induced faster TBARS development at pH 6 than the other hemoglobins (**Figure 3a**). The smaller painty odor changes caused by flounder hemoglobin were not accompanied by lower amounts of deoxyhemoglobin (**Table 1**) or by slower autoxidation of the hemoglobin molecule (**Table 2**). This indicates other characteristics explaining its lower pro-oxidative capacity. The high efficiency of menhaden hemoglobin to catalyze lipid oxidation (**Figures 2a** and **3a**) and to become autoxidized (**Figure 5**; **Table 2**) was also not supported by high initial levels of deoxyhemoglobin (**Table 1**). This stresses that the differences among species regarding hemoglobin deoxygenation were too small to influence lipid oxidation catalysis and hemoglobin autoxidation. This was in contrast to the oxygenation changes caused by acidification. The only hemoglobin for which data on oxygenation/autoxidation fully "supported" data on lipid oxidation was that from pollock. It was hypothesized that the high efficiency of pollock and mackerel hemoglobins to catalyze lipid oxidation at pH 6 (**Figures 2a** and **3a**) was related to the low met-hemoglobin levels at which their own autoxidation became terminated. These levels were only ~50% of the values at which menhaden and flounder hemoglobin autoxidations leveled off (**Figure 5a**). This might indicate that a large fraction of the pollock and mackerel hemoglobins was converted to the met-form immediately when subjected to the pH 6 buffer, even before the first measurement was done. This would then quickly trigger lipid oxidation.

At pH 7.2, all four of hemoglobins were clearly differentiated in terms of their pro-oxidative activities, with pollock being the most active followed by mackerel, menhaden, and then flounder (**Figures 2** and **3**). Except for pollock, this order was not reflected in the initial levels of deoxyhemoglobin and hemoglobin autoxidation rates, which ranked the species as pollock $>$ mackerel ~ menhaden ~ flounder. This further indicates the involvement of other differences among the hemolysates explaining lipid pro-oxidative differences.

Tentative species differences that are not directly related to the tendency of the heme group to become deoxygenated and oxidized involve the size of the heme pocket in the four hemoglobins, the tendency of met-hemoglobin to liberate heme during oxidation, and the tendency of the hemoglobin tetramer to dissociate into subunits (*34*). In addition to their more easily oxidized character (*32*), dimers and monomers are less bulky, which could facilitate reactions with lipid hydroperoxides.

In addition to hemoglobin, the erythrocytes contain, for example, antioxidants (*35*). Although very diluted (~280 times), it was evaluated whether a higher content of antioxidants in the erythrocytes of flounder could explain why this hemolysate gave rise to the longest lipid oxidation lag phase at pH 7.2. As shown in **Figure 4**, there was no increase in lipid oxidation after dialysis. The only effect of dialysis was that the flounder hemolysate yielded lower maximum levels of TBARS. Because addition of both hemolysates was carefully adjusted to give the same hemoglobin levels, we believe dialysis reduced or inactivated catalytically active heme.

Biological Reasons for Differences in Stability and Pro-oxidative Activity. On the basis of the finding of Richards and Hultin (*17*) that hemoglobin from herring and mackerel was more pro-oxidative than hemoglobin from trout, it was believed that the frequent migration of herring and mackerel would give rise to more unstable and pro-oxidative hemoglobins. Highly active species are noted for having very strong Bohr effects (*16*). However, this study showed that a nonmigratory species, pollock, had the most active hemoglobin, which indicates that other biological factors might be of greater importance. Rather than "migration habits", adaptation of the four fish species to

different depths is a possible reason the hemoglobins could have different pro-oxidative activities. Wilson and Knowles (*36*) found that oxyhemoglobin from deep-sea fish was more quickly autoxidized than oxyhemoglobin from species living in more shallow waters. On the basis of autoxidation studies at high pressure (5000 psi), it was concluded that the susceptibility to autoxidation was due to adaptation to low temperature rather than to high pressure. It is generally known that heme proteins of fish have a more flexible and unstable protein conformation than warm-blooded animals due to adaptation to cold water temperatures (*37*). One reason for the high pro-oxidative activity of the pollock hemoglobin could thus be its adaptation to the cold conditions found at ∼200 m (*38*). It was also previously seen (C. Thongraung and M. Pazos, 2003, personal communication) that hemoglobin from a related species living under similar conditions, cod, showed very high pro-oxidative activity (*38*). Among the other species tested, flounder usually lives at a ∼5 m depth (*39*), at which the water does not have the same constant low temperature. Mackerel and menhaden are pelagic, that is, surface- to midwater-swimming fish species. Mackerel can, however, go down to 200 m (*40*), whereas menhaden does not usually go below 20 m (*41*). Menhaden is also regarded as a subtropical species and can be adapted to waters with temperatures of up to 26 °C. Menhaden caught along the coast of New England usually come as adults from the warmer waters in the south. This could account for the greater stability of its hemoglobin as compared to that for mackerel observed in this study. Other recent findings further support that temperature adaptation is important for the pro-oxidative properties of hemoglobins. Hemolysates from a tropical fish (tilapia, *Oreochromis niloticus*) had a low pro-oxidative activity compared to winter flounder (*Pleuronectes americanus*) (H. G. Kristinsson, unpublished data). Also, hemolysates from trout, chicken, and beef were ranked as follows in terms of both lipid oxidation catalysis and degree of deoxygenation: trout ≫ chicken > beef (*42*).

It is not unlikely that some of the differences seen between the four hemoglobins with regard to their efficiency in catalyzing lipid oxidation were due also to other factors than the species origin. One such factor is age. On the basis of size (5 and 20 cm, respectively), the menhaden and pollock used were probably very young. For salmonoid species, it has been described how the ratio between cathodic and anodic hemoglobins increases with age (*43*), which would raise the stability.

From this paper, it is evident that pH reductions within the range that can be seen in post-mortem fish muscle catalyze the pro-oxidative properties of fish hemoglobins. It is also evident that pelagic migratory fish may not necessarily have more lipid pro-oxidative hemoglobin because of their need for oxygen to carry out long-distance migratory swimming. Other factors, such as adaption to greater depths/water temperatures, seem to be more important on the basis of the high catalytic activities of pollock hemoglobin, followed by mackerel, menhaden, and then flounder hemoglobin.

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