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Deoxyhemoglobin-Mediated Lipid Oxidation in Washed Fish Muscle

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Deoxyhemoglobin-mediated lipid oxidation was studied by comparing the pro-oxidative activity of anodic and cathodic hemoglobins from trout in a washed cod muscle model system. At pH 6.3, cathodic hemoglobins were nearly fully oxygenated while anodic hemoglobins were poorly oxygenated. Anodic hemoglobins initiated lipid oxidation in washed cod muscle much more rapidly than cathodic hemoglobins, as measured by thiobarbituric acid reactive substances (TBARS) formation. Moreover, anodic hemoglobins appeared to oxidize more rapidly as compared to cathodic hemoglobins in the washed cod muscle model system, as measured by a decrease in redness (a* value). A more pronounced pro-oxidative activity of deoxyhemoglobin as compared to oxyhemoglobin was confirmed by accelerated lipid hydroperoxide and TBARS formation in the washed cod muscle model system upon combined addition of anodic hemoglobins and adenosine triphosphate, which is known to lower the oxygenation of anodic hemoglobins at pH 7.2, as compared to only addition of anodic hemoglobins to the washed cod muscle. These studies suggest that deoxyhemoglobin is more pro-oxidative than its oxygenated counterpart at pH values found in postmortem fish muscle.

KEYWORDS: Deoxyhemoglobin; hemoglobin oxygenation; blood; quality deterioration; washed fish muscle; lipid oxidation

INTRODUCTION

Hemoglobin is known to be an effective catalyst of lipid oxidation (*1*). Lipid oxidation is a significant problem in the food industry, as it often contributes to odor and flavor deterioration, which lowers the value of the foods (*2*). Numerous pathways by which hemoglobin promotes lipid oxidation have been proposed. One includes the formation of a ferryl protein radical that can initiate lipid oxidation (*3*). The ferryl protein radical forms upon reaction of methemoglobin with either hydrogen peroxide or lipid hydroperoxides. Other pathways of lipid oxidation mediated by hemoglobin include the action of hemin or iron that become released from the heme protein (*2*, *4*). However, the relative contributions of different forms of hemoglobin have not yet been definitively assigned.

Relatively few studies have focused on the pro-oxidative activity of the deoxy species of hemoglobins even though deoxyhemoglobin has been proposed to accelerate lipid oxidation. Pietrzak and Miller (*5*) investigated the ability of oxyhemoglobin, deoxyhemoglobin, and methemoglobin to stimulate lipid oxidation in egg lecithin liposomes. Using numerical integration techniques from oxygen consumption and methemoglobin formation data, these authors found that deoxyhemoglobin was around 3.5 times more capable of stimulating lipid oxidation than methemoglobin and oxyhemoglobin.

It might be assumed that the levels of deoxyhemoglobin are miniscule in aerobic atmospheres. However, the reductive potential inside muscle-based foods keeps most heme compounds in their reduced state during the early periods of storage. Moreover, the oxygenation of certain fish hemoglobins decreases sharply when pH is reduced from 7.5 to 6.5 at atmospheric oxygen pressure, as shown for trout hemoglobins (*6*). When pH was reduced from 7.6 to 6.0, oxygenation of trout hemoglobins decreased with a simultaneous increase in lipid oxidation rates in washed cod muscle suggesting a possible role of deoxyhemoglobin as an effective catalyst of lipid oxidation (*7*). pH values of around 6.0 are typically found in postmortem muscle tissue. Rainbow trout possess anodic and cathodic hemoglobins also called acidic and basic hemoglobins, respectively (*8*). The anodic hemoglobins bind oxygen poorly at pH values around 6 while cathodic hemoglobins retain strong binding of oxygen independent of pH (*8*). Anodic and cathodic hemoglobins comprise around 60 and 40% of the total hemoglobin in rainbow trout, respectively (*9*). The objective of the present study was to examine the relevance of deoxyhemoglobin as a catalyst of lipid oxidation using trout hemoglobins and washed cod muscle as a model system.

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MATERIALS AND METHODS

Chemicals. Bovine hemoglobin, adenosine triphosphate (ATP), streptomycin sulfate, sodium heparin, ferrous sulfate, barium chloride, and Tris[hydroxymethyl]aminomethane (Tris) were obtained from Sigma Chemical A/S (St. Louis, MO); tetraethoxypropane and tetrabutylammonium hydrogen sulfate were obtained from Merck (Hohenbrunn, Germany); and ammonium thiocyanate and trichloroacetic acid (TCA) were obtained from Riedel-deHaën (Seelze, Germany). All other chemicals used were of analytical grade, and double deionized water was used throughout.

Blood Collection. Rainbow trout were obtained from Mølle Fisk (Ravenstrup, Denmark). The fish were anesthetized by electrical stunning. Approximately 4 mL of blood was drawn from the caudal vein (*10*) via syringe, and a 25G needle was preloaded with 1 mL of 150 mM NaCl and sodium heparin (120 Units/ml).

Preparation of Hemolysate. The method of Fyhn et al. (*11*) was used with slight modifications. Four volumes of ice cold 1.7% NaCl in 1 mM Tris, pH 8.0, was added to heparinized blood and centrifuged (700*g* for 10 min at 4 °C) in an IEC Centra MP4R centrifuge (Needham, MA). After the plasma was eliminated, the red blood cells were washed by suspending 3 times in 10 volumes of the above buffer. Cells were lysed in 3 volumes of 1 mM Tris, pH 8.0, for 1 h. One-tenth volume of 1 M NaCl was then added to aid in stromal removal before ultacentrifugation (28 000*g* for 15 min at 4 °C) using a 2331 Ultraspin 70 (LKB, Bromma, Sweden).

Preparation of Anodic and Cathodic Hemoglobins. Hemolysate was transferred to 50 mM Tris, pH 8.6, buffer using PD-10 columns (Bio-Rad, Hercules, CA) and simultaneously separated from low molecular weight components (<10 kDa). Columns (20 mL, 1.5 cm diameter) (Bio-Rad, Hercules, CA) containing degassed DEAE anion exchange chromatography resin were equilibrated with 20 mM Tris buffer (pH 8.6). Initially, cathodic hemoglobins were eluted with 20 mM Tris, pH 8.6. Subsequently, anodic hemoglobins were washed out using 20 mM Tris buffer (pH 8.6) containing 0.5 M NaCl. Finally, both anodic and cathodic hemoglobins were dialyzed extensively (4 °C) to exchange buffer that was 1 mM Tris buffer (pH 8.0).

Measuring the Relative Oxygenation of Hemoglobins. Solutions containing hemoglobin were scanned from 630 to 500 nm using a HP 8452 UV−vis diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA). The blank contained only buffer. The absorbance at the peak (575 nm) minus the absorbance at the valley (560 nm) was calculated. Larger differences indicated that the hemoglobin was more highly oxygenated (*12*). These experiments were run at atmospheric conditions. The percentages of oxygenated molecules were estimated using equations described by Benesch et al. (*13*).

Washed Cod Matrix. Whole cod fish obtained from the local fish market were hand-filleted and skinned. All dark muscle was removed. The rest of the fillets were ground in a mechanical mincer model TS12E (Omas Food Machinery, Oggiona S. Stefano, Italy) (plate diameter 3 mm). The mince was washed twice in distilled deionized water at a 1:3 mince-to-water ratio (w:w) by stirring with a plastic rod for 2 min. Subsequently, the mixture was allowed to stand for 15 min before dewatering through four layers of cheesecloth. The mince was then mixed with 50 mM sodium phosphate buffer (pH 6.3) at the same 1:3 ratio and homogenized using an Ultra Turrax T25 (Staufer, Germany) for 1 min (medium speed). It was allowed to stand for 15 min and finally centrifuged (15 000*g* for 20 min at 4 °C) using a 2331 Ultraspin 70 (LKB, Bromma, Sweden). Minced cod was stored at 4 °C for no longer than 24 h before use.

Quantifying Hemoglobin Levels. The absorbance at 525 nm of hemoglobin solutions in 1 mM Tris, pH 8.0, was determined using a HP 8452 UV−vis diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA). An extinction coefficient of 2.58×10^4 L/cm^{-1} M^{-1} was used to quantify hemoglobin concentrations (*14*). A standard curve was constructed using bovine oxyhemoglobin standard (Sigma) in 1 mM Tris, pH 8.0, buffer.

Addition of Hemoglobins and ATP to Washed Cod Muscle. An appropriate volume of the hemoglobin stock was added to a final concentration of 5.8 μmol per kg washed cod muscle and stirred with a plastic spatula for 3 min to distribute the heme protein. Streptomycin sulfate (200 ppm) was added to inhibit microbial growth during storage. The pH of the samples was checked just after the addition of hemoglobin, periodically during storage, and finally at the end of storage. The final moisture content of the samples stored at 2 °C was adjusted to 88%. A stock solution of ATP (500 mM) in distilled deionized water was adjusted to pH 7.2 with 1 N NaOH prior to adding to a final concentration of 5 mM in washed cod muscle.

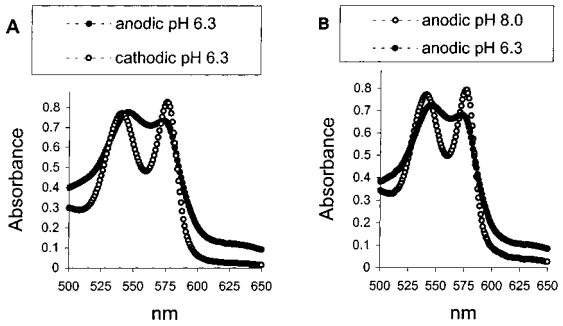

Figure 1. (**A**) Spectra of anodic and cathodic hemoglobins at pH 6.3. (**B**) Spectra of anodic hemoglobins at pH 6.3 and after increasing pH back up to 8.0 with NaOH. A deeper trough at 560 nm is indicative of increased oxygenation (*12*).

Determination of Thiobarbituric Acid Reactive Substances (TBARS). TBARS were determined according to a modified procedure of Buege and Aust (*15*). Fifty percent TCA containing 1.3% thiobarbituric acid (TBA) was heated to 65 °C on the day of use to dissolve the TBA. Approximately 150 mg of sample was added to 1.1 mL of the TCA−TBA mixture and incubated for 1 h at 65 °C. After centrifugation (2500*g* for 10 min), the absorbance at 532 nm of the supernatant was determined. A standard curve was constructed using tetraethoxypropane.

Determination of Lipid Hydroperoxides. Between 0.4 and 0.5 g of washed cod muscle was homogenized in 5 mL of chloroform/methanol (1:1) for 30 s. Subsequently, the polytron was washed for 30 s with 5 mL solvent, which was finally mixed with the 5 mL of homogenate. Three milliliters of 0.5% NaCl was added, and the mixture was vortexed for 30 s before centrifugation for 10 min to separate the mixture into two phases. A 1.33 mL amount of ice cold chloroform/methanol (1:1) was added to 2 mL of the lower phase and vortexed briefly. A total of 25 μL ammonium thiocyanate (4.38 M) and 25 μL iron(II)chloride (18 mM) were added to the assay for lipid hydroperoxides (*16*), and samples were incubated for 20 min at room temperature before the absorbances at 500 nm were determined. A standard curve was prepared using cumene hydroperoxide. The used chloroform included ethanol as a preservative to omit high blank readings, as described previously (*17*).

Color Measurements. The a values were measured with a Minolta CR-200 chroma meter (Minolta Camera Co., Osaka, Japan). A white calibration plate supplied with the unit was used to calibrate the instrument.

Statistical Evaluations. All experiments were done at least in duplicate. A linear regression procedure of the SAS system was used to evaluate data from storage studies (*18*). Data from the entire storage period for each type of sample were grouped prior to making comparisons.

RESULTS

Visible spectra of the anodic and cathodic hemoglobins from rainbow trout showed that cathodic hemoglobins are much more oxygenated at pH 6.3 as compared to anodic hemoglobins, which contained large amounts of deoxyhemoglobin (**Figure 1A**). The larger valley at around 560 nm in the cathodic hemoglobins is indicative of higher oxygenation (*12*). Anodic and cathodic hemoglobins were around 67 and 5% deoxygenated at pH 6.3, respectively. Anodic hemoglobins became more oxygenated when the pH was increased to pH 8.0 with NaOH

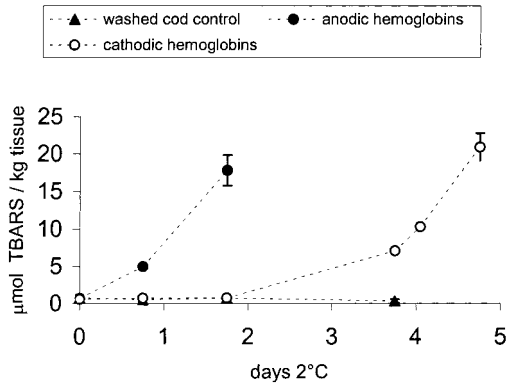

Figure 2. TBARS formation due to anodic and cathodic hemoglobins added separately to washed cod muscle at pH 6.3 during 2 °C storage. Hemoglobin concentration was 5.8 μmol per kg washed cod muscle.

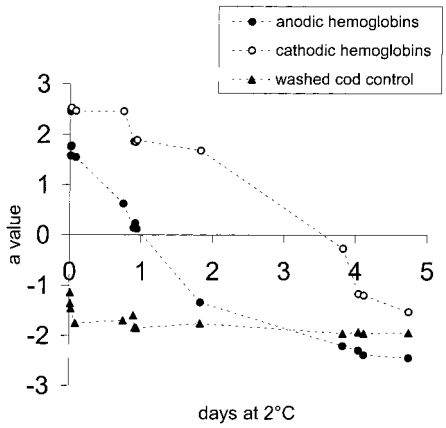

Figure 3. Changes in a values (redness) during 2 °C storage of washed cod muscle containing anodic or cathodic hemoglobins. The pH of the samples was 6.3. Hemoglobin concentration was 5.8 μmol per kg washed cod muscle.

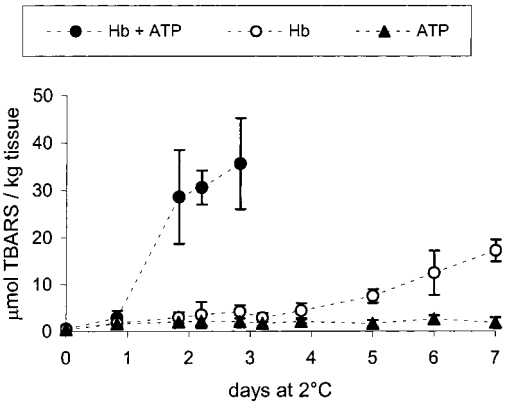

Figure 4. Effect of 5 mM ATP on anodic hemoglobin-mediated oxidation of washed cod tissue at pH 7.2 during 2 °C storage. TBARS were used as the indicator of lipid oxidation. Hemoglobin concentration was 5.8 μmol per kg washed cod muscle.

and subsequently less oxygenated when the pH was regulated back to 6.3 (**Figure 1B**). This indicates reversibility in oxygenation of anodic hemoglobins upon pH adjustment. It is clear that cathodic hemoglobins do not become less oxygenated at the reduced pH.

Anodic and cathodic hemoglobins at a final concentration of 5.8 μmol/kg washed cod muscle were added separately to washed cod muscle and adjusted to pH 6.3 to assess the ability of each hemoglobin species to initiate lipid oxidation. pH 6.3 was chosen since this is a typical postmortem pH value found in many fish muscles and because anodic hemoglobins at this pH are largely deoxygenated as compared to cathodic hemoglobins, which are highly oxygenated. Washed cod muscle contains a low amount of residual heme pigments and carotenoids as compared to washed trout muscle. Because the residual pigments would likely confound the effect of the added anodic and cathodic hemoglobins, washed cod muscle was used. **Figure 2** shows that anodic hemoglobins initiated lipid oxidation much more rapidly than cathodic hemoglobins during 2 °C storage ($p < 0.01$), as indicated by the increase in TBARS. After around 2 days, TBARS from samples containing anodic hemoglobins surpassed a threshold of 15 μmol/kg washed cod whereas cathodic hemoglobins did not reach this threshold until after 4 days. Washed cod muscle without added hemoglobins did not show any substantial increase in TBARS during 4 days of storage at 2 °C.

The change in redness was determined on the above samples that were also analyzed for the formation of TBARS during storage. At zero time, washed cod muscle samples containing cathodic hemoglobins were more red as compared to equal samples with added anodic hemoglobins, due to the higher oxygenation of cathodic hemoglobins (**Figure 3**). As also seen in **Figure 3**, the loss in red color was more rapid in samples containing anodic hemoglobins as compared to cathodic hemoglobins.

To support whether the above data really show that deoxyhemoglobin is a better initiator of lipid oxidation, rather than a difference in rate of lipid oxidation due to differences in amino acid sequence of anodic and cathodic hemoglobins and thereby different exposure of catalytic site to the environment, the following experiment was performed. It is well-known that the addition of ATP at pH 7.2 lowers oxygenation of trout hemoglobins (*7*) and snake hemoglobins (*19*). **Figure 4** shows that the addition of 5 mM ATP added to anodic hemoglobins accelerated the rate of lipid oxidation in the washed cod muscle system at pH 7.2 ($p < 0.05$). After 2 days at 2 °C, the samples with hemoglobin and ATP showed substantial formation in TBARS as compared to either the samples with only added anodic hemoglobins or the controls. Even after 5 days of storage, TBARS were lower in samples containing only hemoglobin as compared to samples with both hemoglobins and ATP added after 2 days of storage. **Figure 5** shows the results of an additional experiment using ATP to lower oxygenation of anodic hemoglobins at pH 7.2 where the formation of both lipid hydroperoxides and TBARS was determined. In accordance with the above data, a decrease in hemoglobin oxygenation by the addition of ATP accelerated lipid oxidation as compared to washed cod samples that contained only anodic hemoglobins or only ATP based on TBARS ($p < 0.05$) and lipid hydroperoxide formation ($p < 0.05$).

Figure 6 shows the development in TBARS formation in the washed cod muscle system containing either anodic or cathodic hemoglobins at pH 7.4 and pH 6.3. TBARS formed more rapidly in samples containing anodic hemoglobins as compared to cathodic hemoglobins at pH 6.3 ($p < 0.01$) and pH 7.4 ($p < 0.05$). At pH 7.4, the formation of TBARS was slowed considerably independent of the presence of cathodic or anodic hemoglobins.

DISCUSSION

Little has been done in terms of examining the role of heme protein oxygenation on the oxidative stability of lipids in muscle foods. The anodic and cathodic hemoglobins of trout are useful

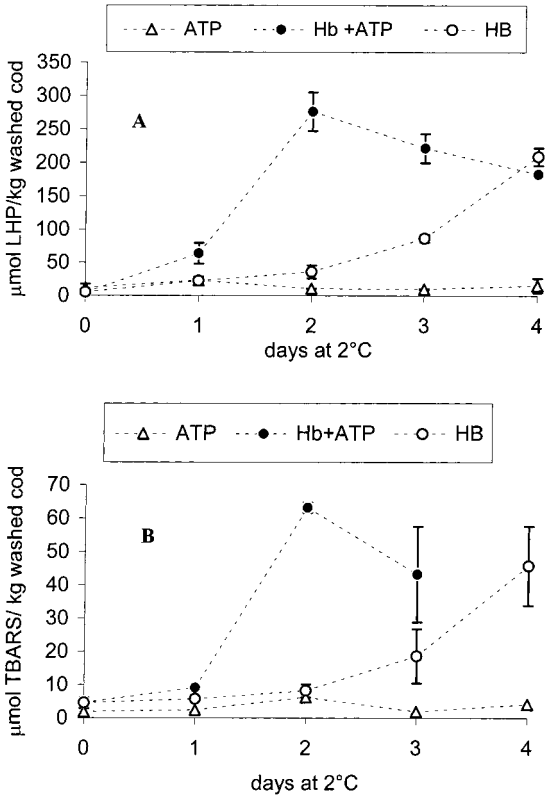

Figure 5. Lipid hydroperoxide (**A**) and TBARS (**B**) development due to anodic hemoglobins added to washed cod in the presence and absence of 5 mM ATP. Hemoglobin concentration was 5.8 μmol per kg washed cod muscle. The pH of the samples was 7.2.

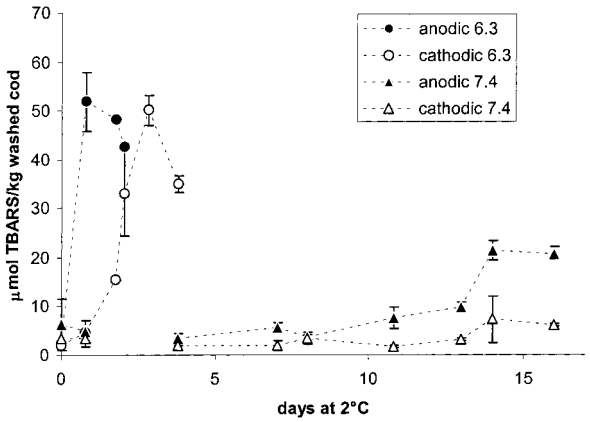

Figure 6. TBARS formation due to anodic and cathodic hemoglobins added separately to washed cod muscle at pH 6.3 and pH 7.4 during 2 °C storage. Hemoglobin concentration was 5.8 μmol per kg washed cod muscle.

in this endeavor since the anodic hemoglobins are poorly oxygenated at pH values found in postmortem muscle while the cathodic hemoglobins are nearly fully oxygenated at postmortem pH values (**Figure 1**). Anodic hemoglobins from trout have been found to be approximately 15% oxygenated in the pH area 6.2−6.5 at atmospheric pressure, while these hemoglobins are more than 95% oxygenated at pH 7.5 (*6*). The decrease in hemoglobin oxygenation with increasing H^+ is known as the Bohr effect (*20*). Thus, anodic hemoglobins exhibit the Bohr effect while cathodic hemoglobins do not exhibit the Bohr effect as is also the case with the muscle pigment myoglobin (*20*).

Hemoglobins with low oxygen affinity are noted for rapid autoxidation rates (*21*), which is relevant in the present context, as autoxidation results in the formation of methemoglobin and the superoxide anion radical ($O_2^{\bullet -}$) in eq 1

$$\mathrm{Hb{-}O_2} \rightarrow \mathrm{MetHb(III)} + \mathrm{O_2^{\bullet -}} \qquad (1)$$

$O_2^{\bullet -}$ dismutates to H_2O_2, which subsequently may react with methemoglobin to form the hypervalent ferrylhemoglobin radical known to initiate lipid oxidation (eq 2) (*22*).

$$\mathrm{MetHb(III)} + \mathrm{H_2O_2} \rightarrow \mathrm{Hb^{\bullet +}(IV){=}O} + \mathrm{H_2O} \qquad (2)$$

Consequently, the increased pro-oxidative activity of anodic hemoglobins in the washed cod muscle system (**Figure 2**) may be described by enhanced autoxidation as compared to more slow autoxidation of cathodic hemoglobins. The accelerated loss in red color in the washed cod muscle matrix with added anodic hemoglobins as compared to cathodic hemoglobins (**Figure 3**) may support such a mechanism. However, present data cannot exclude that the loss in red color is a result of general hemoglobin denaturation or heme destruction due to the accelerated oxidative processes in the washed cod muscle system.

The reason that poorly oxygenated hemoglobins autoxidize faster than highly oxygenated hemoglobins involves the spin state of the iron atom inside the heme ring (*23*). The ferrous iron atom of deoxyhemoglobin (Fe^{2+}) is a 5-coordinated complex where the iron has 4 bonds to the porphyrin heme ring and 1 bond to a histidine residue of the globin. This causes the iron to be in a high spin state and hence highly susceptible to oxidation to ferric methemoglobin (Fe^{3+}). The iron atom of oxyhemoglobin (Fe^{2+}) is a 6-coordinated complex with an additional ligand to O_2, which causes the iron to be in a low spin state and less susceptible to oxidation. A more rapid formation of methemoglobin from deoxygenated molecules likely increases the ability of the heme protein to promote lipid oxidation.

Released hemin from hemoglobin has the ability to stimulate lipid oxidation (*24*, *25*) as it catalyzes the breakdown of preformed lipid hydroperoxides and thereby initiates the production of alkoxyl radicals, which are capable of abstracting a hydrogen atom from polyunsaturated fatty acids with subsequent propagation of lipid oxidation processes. The release of the heme porphyrin from the globin is due to disturbances in a number of stabilizing factors, including hydrophobic, covalent, and electrostatic interactions, of importance for the porphyrin/globin configuration (*26*). There is also evidence that methemoglobin, hemochromes, and hemichromes precede formation of free hemin (*25*, *27*−*29*). Because of fewer stabilizing interactions between the porphyrin structure and the globin moiety in deoxyhemoglobin as compared to the other hemoglobin species (*23*, *30*), anodic hemoglobins might be expected to be more sensitive to hemin release as compared to the more highly oxygenated cathodic hemoglobins at the pH of these studies. Consequently, an accelerated release of hemin could explain the more pro-oxidative activity of the deoxy forms in the present system.

Deoxygenated hemoglobin is less compact than oxyhemoglobin, and the iron atom moves out of the plane of the heme ring (*20*). Moreover, deoxygenated hemoglobins have greater heme pocket flexibility (*31*). These differences in quaternary structures may allow better access of fatty acid or lipid hydroperoxides to the heme crevice of the deoxy as compared to the oxy forms and thereby stimulate lipid oxidation, as structurally changed myoglobins, where better access of lipid

substrate to the heme crevice has been shown to increase peroxidation of linoleic acid as compared to the native myoglobin (*32*).

The present study also showed that ATP accelerated anodic hemoglobin-mediated lipid oxidation in the washed cod muscle, as a consequence of the ability of ATP to lower oxygenation of anodic hemoglobins. ATP is present in muscle tissue in vivo at high concentrations (5−10 mM) and is metabolized during the postmortem period. There have been reports that lipid oxidation occurs more rapidly in processed muscle from fresh fish as compared to older fish (*33*−*35*). This may solely be due to the amount of residual ATP, as ATP in older fish is depleted at the time of mincing, and thereby does not favor deoxyhemoglobin formation upon mincing, which mechanically facilitates the release of hemoglobin from capillaries and erythrocytes.

TBARS formed more rapidly in samples containing anodic hemoglobins as compared to cathodic hemoglobins at pH 7.4 ($p < 0.05$). Harrington et al. (*8*) showed that anodic hemoglobins of trout were slightly less oxygenated at pH 7.4 using a dissociation analyzer. This can partially explain the more rapid TBARS formation by anodic hemoglobins at pH 7.4. At pH 6.3, it might be expected that a much greater difference in the lipid oxidation rate would occur between anodic and cathodic hemoglobins as compared to pH 7.4 since there is a much greater difference in oxygenation between the two hemoglobins at pH 6.3. However, acid-catalyzed autoxidation of hemoglobin will be more rapid at pH 6.3 as compared to pH 7.4 (*36*). Thus, the acid-catalyzed autoxidation of both anodic and cathodic hemoglobins will have more of an effect at pH 6.3 than pH 7.4, which will lessen the observable effect of the larger difference in oxygenation between anodic and cathodic hemoglobins at pH 6.3 as compared to pH 7.4.

Finally, an increase in pH from 6.3 to 7.4 decreased the rate of lipid oxidation considerably independent of whether anodic or cathodic hemoglobins were added to washed cod muscle (**Figure 6**). That cathodic hemoglobins became much more pro-oxidative at lower pH despite the same degree of oxygenation must be explained by a direct pH effect. Low pH is known to enhance (i) hemin release (*37*), (ii) solubility of iron released from hemoglobin (*38*), (iii) acid-catalyzed hemoglobin autoxidation (*36*), and (iv) formation of H_2O_2 from $O_2^{\bullet -}$ (*39*), which are all parameters known to stimulate lipid oxidation (*2*, *4*).

In conclusion, the present data show that deoxyhemoglobin is more pro-oxidative than oxyhemoglobin in washed cod muscle; however, whether this is due to (i) more readily formed ferrylhemoglobin, (ii) release of hemin, (iii) better access of lipid hydroperoxides to the heme crevice in deoxyhemoglobin, or (iv) a combination of these mechanisms cannot be concluded from the present data and needs further attention in future studies.

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