Effect of pH on Lipid Oxidation Using Trout Hemolysate as a Catalyst: A Possible Role for Deoxyhemoglobin

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Hemoglobin-mediated lipid oxidation was studied by adding hemolysate to washed cod muscle. Three pH values were examined (pH 7.6, 7.2, and 6.0). The lag time prior to rancidity and thiobarbituric acid reactive substance development decreased greatly as the pH was reduced ($p < 0.01$). Formation of methemoglobin due to autoxidation of the heme pigment was found to occur more rapidly at reduced pH. Also, the level of deoxyhemoglobin was found to sharply increase with pH reduction in the range of pH 7.6−6.0. This suggested a potential role for deoxyhemoglobin as a catalyst. ATP lowered hemoglobin oxygenation at pH 7.2. Peroxidation of linoleic acid by oxy/deoxyhemoglobin and methemoglobin was investigated at two levels of preformed lipid hydroperoxides. At a reduced level of preformed lipid hydroperoxides, oxy/deoxyhemoglobin stimulated peroxidation of linoleic acid, whereas methemoglobin did not. At the higher level of preformed lipid hydroperoxides, both oxy/deoxyhemoglobin and methemoglobin were active. This investigation suggests that reduced hemoglobins played an important role in lipid oxidation processes.

Keywords: *pH effect on lipid oxidation; oxygenation of hemoglobin; trout blood; hemolysate; deoxyhemoglobin; methemoglobin; preformed lipid hydroperoxides*

INTRODUCTION

Lipid oxidation is a major cause of quality deterioration in muscle-based foods where flavor, color, texture, and nutritional value can be negatively affected (Kanner, 1994; Gandemer and Meynier, 1995). Heme pigments such as hemoglobin and myoglobin are believed to contribute to lipid oxidation of tissue (Koizumi et al., 1987; Chan et al., 1997). It was reported that human hemoglobin stimulated peroxidation of linoleic acid only slightly at pH 7.4, but the rate increased considerably at pH 6.5 (Gutteridge, 1987). Values of pH below neutrality are typical of post mortem muscle systems. Thus, knowledge about the role of pH below neutrality is critical in understanding lipid oxidation in post mortem meat and fish.

Acceleration of lipid oxidation by pH reduction could be due to enhanced autoxidation of hemoglobin at reduced pH (Tsuruga et al., 1998). Hemoglobin autoxidation causes the production of superoxide anion radical and methemoglobin from ferrous oxyhemoglobin (Misra and Fridovich, 1972). Dismutation of superoxide anion radical will produce hydrogen peroxide, which activates methemoglobin as an initiator of lipid peroxidation (Harel and Kanner, 1986). Lowering the oxygenation of hemoglobin was found to enhance the autoxidation of hemoglobin (Balagopalakrishna et al., 1996). This is relevant since binding of oxygen by hemoglobins in rainbow trout decreased as pH was lowered (Binotti et al., 1971). Many fish species have multiple hemoglobin components (Zolese et al., 1999). Acidic and basic hemoglobins have been identified in salmon, herring, and sardines (Harrington, 1986; Rizzotti and Gioppato, 1999). Binding of oxygen by acidic hemoglobins is dependent on pH, whereas basic hemoglobins bind oxygen independent of pH. Thus, lowering pH will lower hemoglobin oxygenation of acidic hemoglobins, which could promote lipid oxidation via acceleration of hemoglobin autoxidation. The major hemoglobin of rainbow trout, component IV, is an acidic hemoglobin (Zolese et al., 1999).

At the same time, there is evidence that formation of methemoglobin may not be necessary for lipid peroxidation to occur (Tappel, 1955). This author suggested that reduced hemoglobins did not undergo a valency shift during catalysis of linoleate oxidation since catalysis occurred in the presence of carbon monoxide, which combines strongly with the ferrous ions in the hemoglobin.

A quasi-lipoxygenase activity of hemoglobin has been described (Kuhn et al., 1981; Everse and Hsia, 1997). If hemoglobin acts as an enzyme, changes in the electrostatic charge of amino acids due to pH adjustment may affect catalysis of lipid peroxidation at the active site. The main objective of this work was to investigate the effect of pH on lipid oxidation in washed minced cod muscle caused by trout blood hemolysate as a source of hemoglobin.

MATERIALS AND METHODS

Fish Supply. Rainbow trout (*Onchorhynchus mykiss*) (25−31 cm) were supplied by the Mohawk Trout hatchery (Sunderland, MA). Trout were maintained in an opaque, plastic tank (Nalgene, Rochester, NY). Freshwater was used that was constantly circulated with a Whisper power filter, Turbo 5 (Willinger Bros. Inc., Oakland, NJ), equipped with disposable Bio-Bags to collect waste and ammonia. Fish were fed Silver Cup trout chow (Nelson's Sterling, Murray, UT), 1 g per fish per day. The water temperature was maintained at 4−8 °C.

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10.1021/jf991059w CCC: $19.00 © 2000 American Chemical Society
Published on Web 07/14/2000

Bleeding of Trout. Trout were anesthetized in 3-aminobenzoic acid ethyl ester (0.5 g/L) for 3 min. The fish was then held belly up, and 1 mL of blood was drawn from the caudal vein (Rowley, 1990) with a syringe and 25 gauge needle (Becton Dickinson, Franklin Lakes, NJ) preloaded with 1 mL of salined sodium heparin (30 units/mL). Hemolysate was prepared immediately after the blood was drawn.

Preparation of Hemolysate. The method of Fyhn et al. (1979) was modified. Four volumes of ice cold 1.7% NaCl in 1 mM Tris, pH 8.0, were added to heparinized blood. Centrifugation was done at 700*g* for 10 min at 4 °C using a tabletop clinical centrifuge (IEC, Needham Heights, MA). Plasma was then removed. Red cells were washed by suspending three times in 10 volumes of the above buffer and centrifuging at 700*g*. 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 centrifugation at 28000*g* for 15 min at 4 °C in a chilled no. 40 rotor in a Beckman Ultracentrifuge model L5-65B (Beckman Instruments Inc., Palo Alto, CA). The hemolysate was stored on ice and used within 2 h.

Quantifying Hemoglobin Levels in Hemolysate. The method of Brown (1961) was adapted. The hemolysate was diluted in 50 mM Tris, pH 8.6. Diluted hemolysate was poured into a 1.5 mL disposable cuvette. Around 1 mg of sodium dithionite was added to the extract and mixed. Then the sample was bubbled with carbon monoxide gas (Matheson Gas, Gloucester, MA) for 30 s. The sample was then scanned from 440 to 400 nm (Soret) against a blank that contained only buffer using a model U-3110 double-beam spectrophotometer (Hitachi Instruments, Inc., San Jose, CA). The peak absorbance was recorded. Standard curves were constructed using bovine hemoglobin standard (Sigma, St. Louis, MO). An extinction coefficient of 4.60×10^5 L/cm mol was determined.

Washed Cod Matrix. Whole cod fish was hand filleted and skinned. All dark muscle was removed. The rest of the fillets were minced in a Kitchen Aid (Kitchen Aid Inc., St. Joseph, MI) mincer (diameter 4.7 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, allowing the mixture to stand for 15 min and then dewatering on a fiberglass screen. It was then mixed with 50 mM sodium phosphate buffer (pH 6.0, 7.2, or 7.6) at the same 1:3 ratio and homogenized using a Polytron (Brinkman Instruments, Westbury, NY) for 1 min (rheostat at 30). It was allowed to stand for 15 min and then centrifuged at 15000*g* for 20 min at 4 °C in a Beckman Ultracentrifuge model L5-65B (Beckman Instruments Inc., Palo Alto, CA).

Hemoglobin Levels Added to the Washed Cod. A volume of hemolysate sufficient to provide hemoglobin at the stated concentration was delivered into the washed cod mince. Samples contained 200 ppm streptomycin sulfate to inhibit microbial growth. The pH of the samples was checked just after hemolysate was added and at the end of storage. The final moisture content of the samples that were stored at 2 °C was adjusted to 90%.

Preparation of Methemoglobin. Methemoglobin was prepared by adding a 3-fold molar excess (heme basis) of $K_3Fe(CN)_6$ to freshly prepared hemolysate for 30 min on ice (DeYoung et al., 1994). Removal of unreacted ferricyanide and ferrocyanide was accomplished with gel filtration using Bio-gel p-6DG gel in 10DG columns (Bio-Rad, Hercules, CA). Reduced hemoglobins were subjected to gel filtration when compared to methemoglobin.

Sensory. Five to eight trained panelists (Richards et al., 1998) sniffed samples contained in 30 mL capacity brown bottles with caps. Panelists concentrated on detecting rancid/painty odors using a scale of 0−10, with 10 being the strongest. Samples were stored at 2 °C for up to 15 days.

Thiobarbituric Acid Reactive Substances. Thiobarbituric acid reactive substances (TBARS) were determined according to the method of Lemon (1975). A modification included using 1 g of sample and extracting with 6 mL of TCA by homogenization with a Tissue Tearor model 986-370 at high speed (Biospec Products, Racine, WI).

Measuring the Relative Oxygenation of Hemoglobin. Solutions containing hemoglobin were scanned from 630 to 500 nm using a double-beam spectrophotometer model U-3110 (Hitachi Instruments, Inc., San Jose, 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. Estimates of oxygenated molecules were based on the spectra of oxyhemoglobin and deoxyhemoglobin from Boyle et al. (1994). These experiments were run at atmospheric conditions.

Reduction of Linoleic Acid Hydroperoxides. The method of Baron et al. (1997) was used to reduce the preformed lipid hydroperoxides that contaminated the linoleic acid substrate. All buffers were passed at least twice through Chelex 100 (Sigma, St. Louis, MO) packed in 20 mL disposable columns (Bio-Rad, Hercules, CA) to remove any free metal ions. Linoleic acid (250 mg) was dissolved in 20 mL of a 1:1 mixture of ethanol and Borax buffer (5 mM, pH 8.8). The pH was adjusted to 9.0, and $NaBH_4$ (100 mg) was added. After 1 h of continuous stirring at room temperature, the mixture was diluted with distilled deionized water (10 mL) and the pH was lowered to approximately 3.0 with HCl (2 M). The mixture was extracted with 2×20 mL of diethyl ether. The ether extract was washed with a saturated solution of sodium bicarbonate (1:1). The ether was dried with $MgSO_4$ and evaporated under a steam of nitrogen.

Measuring Rate of Conjugated Diene Formation during Reaction of Linoleic Acid with Hemoglobin Species. Linoleic acid was diluted to 36.5 mM in sodium phosphate buffer (10 mM, pH 7.2) and Tween 20 (10 mg/mL). This was the substrate solution. The reaction mixture consisted of 0.15 mL of substrate solution, 0.35 mL of sodium phosphate buffer (10 mM, pH 7.2), and 0.50 mL of hemolysate at a hemoglobin concentration of 2 μM in 10 mM sodium phosphate buffer, pH 6.0. Reduced hemoglobins and methemoglobins were evaluated as catalysts. During a 10 min incubation of the reaction mixture at 6 °C, absorbance changes at 234 nm (conjugated dienes) were continuously measured. Using a double-beam spectrophotometer, the rate of conjugated diene formation caused by hemoglobins could be determined directly in the samples. The blank contained all components except hemoglobins.

Determination of Lipid Hydroperoxides. After a 10 min incubation period of the reaction mixture described in the section directly above, 0.8 mL of the reaction mixture was added to 11 mL of chloroform/methanol (2:1). Lipid hydroperoxides were also determined in 0.8 mL of the initial substrate solution. The chloroform used must have ethanol as its preservative since other preservatives caused high blank readings. The mixture was homogenized in a 16×125 mm disposable glass tube with a Tissue Tearor model 986-370 at moderate speed for 30 s. Samples were filtered in a 5 cm^3 Luer-LOK syringe (Becton-Dickinson and Co., Franklin Lakes, NJ) with a 0.7 μm pore size, 13 mm ZC disposable glass fiber filter attached (Whatman Inc., Clifton, NJ). Seven mL of the filtrate was added to a 16×125 mm tube. Two ml of 0.5% NaCl was added. Samples were vortexed at a moderate speed for 30 s and then centrifuged for 3 min in a tabletop centrifuge to separate the sample into two phases. Two mL of ice cold chloroform/methanol (2:1) was added to 3 mL of the lower phase. Ammonium thiocyanate and iron(II) chloride were prepared as in Shantha and Decker (1994). Tweny-five μL of each reagent was added followed by vortexing for 2−4 s. Samples were incubated for 20 min at room temperature. Then absorbance was measured at 500 nm. Samples were kept on ice, leading up to the 20 min incubation step. A standard curve was prepared using cumene hydroperoxide.

Statistical Evaluations. All experiments were done at least twice. An unpaired t-test was used to determine significant differences between samples that did not undergo a storage period (Tilling et al., 1994). A linear regression procedure of the SAS system was used to evaluate data from storage studies (Cody and Smith, 1997).

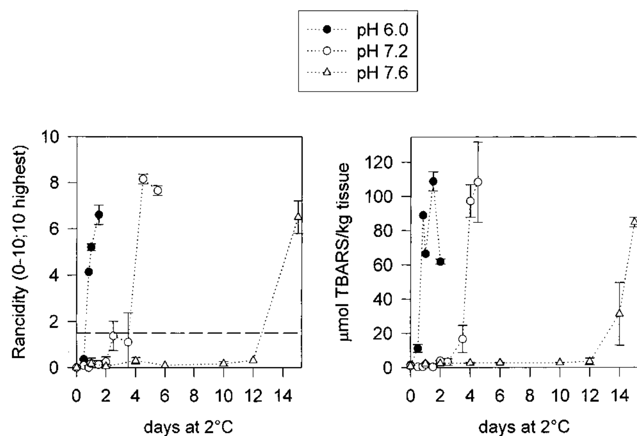

Figure 1. Sensory scores and TBARS of washed cod added to trout hemolysate (5.8 μmol of hemoglobin/kg of tissue) at pH 6.0, 7.2, and 7.6. The dashed horizontal line indicates a slight rancid odor.

RESULTS

Hemolysate was added to washed cod so that the hemoglobin concentration was 5.8 μmol/kg of tissue. This was the approximate level of hemoglobin found in trout whole muscle (data not shown). Trout hemolysate induced lipid oxidation of washed cod muscle more rapidly at pH 6.0 compared to pH 7.2 on the basis of sensory scores and TBARS values (Figure 1). At pH 6.0, rancidity occurred in less than 1 day, whereas, at pH 7.2, rancidity developed after about 3.5 days. Increasing the pH to 7.6 delayed rancidity and TBARS development to a greater extent than pH 7.2 (Figure 1). Linear regression analysis indicated that increasing the pH significantly increased the lag time prior to rancidity ($p < 0.01$) and TBARS ($p < 0.01$) development. The lag time was considered the time it took for TBARS to exceed 20 μmol/kg of tissue and rancidity to exceed a value of 1.5, which is the point at which the sample had a slight rancid odor. Since the trout hemolysate was added to washed cod within a couple of hours of preparation, little methemoglobin was present (on the basis of spectral data, not shown). When methemoglobin was added to washed cod at either pH 6.0 or pH 7.6, rancidity developed within 1 day (data not shown).

It is important to be aware that oxygenation of hemoglobin can decrease as conditions become acidic. This is known as the Bohr effect (Stryer, 1988). Oxygenation of hemoglobin component IV in rainbow trout was highly dependent on pH (Binotti et al., 1971). Component IV is one of the four types of hemoglobin in rainbow trout. At pH 6.5, around 15% of component IV was oxyhemoglobin and 85% was deoxyhemoglobin. At pH 7.5, over 90% was oxyhemoglobin, with the rest being in the deoxyhemoglobin state. Using our diluted trout hemolysate, oxygenation of hemoglobin increased with increasing pH between pH 6.0 and pH 8.0 in a sigmoidal fashion (Figure 2).

The effects of various components on the oxygenation of trout hemoglobin in the hemolysate were compared at pH 7.2 (Table 1). Sodium phosphate buffer and PIPES did not significantly affect oxygenation compared to unbuffered hemolysate (Table 1). On the other hand, ATP and to a lesser extent ADP significantly lowered the oxygenation of hemoglobin at pH 7.2. ATP was shown to lower the oxygen affinity of snake hemoglobin between pH 7.0 and pH 7.4 (Bonafe et al., 1999).

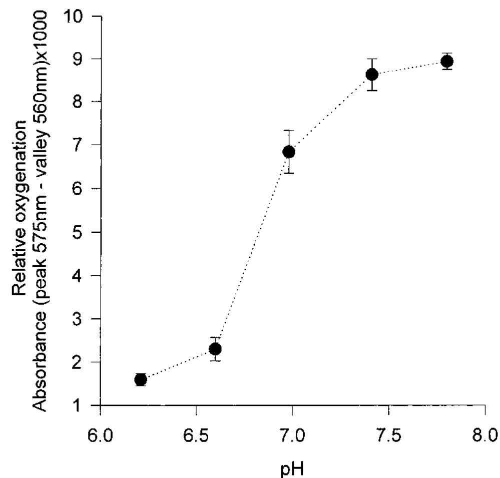

Figure 2. Relative oxygenation of trout hemoglobin from hemolysate diluted in 10 mM sodium phosphate buffer at various pH values. The hemoglobin concentration was 0.5 μM. An increase in oxygenation is indicated by an increase in absorbance.

Table 1. Effect of Various Components on Oxygenation of Trout Hemoglobins at pH 7.2[a]

component added	measure of relative oxygenation (peak abs 575 nm − valley abs 560 nm) × 1000
unbuffered	18.4 ± 2.0 (control)
5 mM ATP	5.9 ± 0.9[b]
5 mM ADP	9.7 ± 1.8[c]
5 mM PIPES	20.2 ± 3.1 (ns)
5 mM sodium phosphate	19.9 ± 3.3 (ns)

[a] The hemoglobin concentration was 0.8 μM. An increased net absorbance value represents increased oxygenation. ns = no significant difference from the unbuffered control. $n = 4$. [b] Very highly significant difference from the control ($p < 0.001$). [c] Highly significant differences from the control ($p < 0.01$).

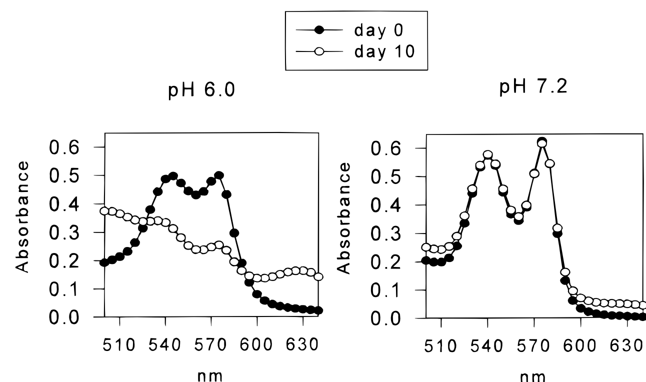

Figure 3. Spectra of freshly prepared and aged hemolysate at pH 6.0 and 7.2. The hemoglobin concentration was 15 μM. The buffer was 10 mM sodium phosphate. The storage temperature was 4−8 °C.

In freshly prepared hemolysate, a deeper trough was observed between 580 and 540 nm at pH 7.2 compared to pH 6.0. In other words, there was a lower absorbance at 560 nm between the two peaks. This trough is characteristic of oxygenated hemoglobin (Figure 3). The hemolysate was stored at 4−8 °C for 10 days at a concentration of 15 μM hemoglobin. At pH 7.2 the spectral characteristics of hemoglobin in the hemolysate changed little during storage. At pH 6.0 the spectra converted to that resembling methemoglobin during the same storage period indicative of substantial autoxidation of the heme protein (Figure 3).

Table 2. Effect of the Oxidation State of Hemoglobin and the Preformed Lipid Peroxide Level in Linoleic Acid Substrate on Lipid Peroxide Formation and the Rate of Development of 234 nm Absorbing Species[a]

sample	preformed lipid peroxides (μM)	ΔA_{234} (nm/min)	lipid peroxide formation (μM)
oxy/deoxyhemoglobin	3.4 ± 0.5	0.51 ± 0.13	165.5 ± 21.3
methemoglobin		0.03 ± 0.02	2.6 ± 1.2
oxy/deoxyhemoglobin	7.9 ± 0.9	0.77 ± 0.18	280.3 ± 47.0
methemoglobin		0.69 ± 0.14	263.3 ± 51.4

[a] The incubation of heme protein and linoleic acid was 10 min at 6 °C. The hemoglobin concentration in the sample was 2 μM, and the final pH was 6.6. Lipid peroxide values reported indicate accumulation after the 10 min incubation period. $n = 4$.

Lipid oxidations due to methemoglobin and oxy/deoxyhemoglobin prepared from trout hemolysate were compared using linoleic acid as substrate. The term oxy/deoxyhemoglobin was used to stress that both components were present since the experimental conditions were at pH 6.6 (Figure 2). The heme proteins were added to linoleic acid solubilized in Tween 20 detergent. Two levels of preformed lipid peroxides were examined. The preformed lipid hydroperoxide content in freshly prepared linoleic acid substrate was 7.9 μM. Sodium borohydride was used to lower the preformed lipid hydroperoxide concentration to 3.4 μM. At the lower level of preformed lipid peroxides, oxy/deoxyhemoglobin catalyzed formation of lipid peroxides as determined by the ferric−thiocyanate assay, whereas methemoglobin did not (Table 2). Conjugated dienes, which absorb at 234 nm, increased rapidly in the samples containing oxy/deoxyhemoglobins but not methemoglobins (Table 2). At the higher level of preformed lipid peroxides, both oxy/deoxyhemoglobin and methemoglobin catalyzed lipid oxidation. Increasing the preformed lipid peroxide level by a factor of 2.3 increased the amount of lipid peroxides formed by oxy/deoxyhemoglobin by a factor of 1.7 (Table 2).

DISCUSSION

There is great interest in understanding the causes of the initial rise in lipid peroxides in foods. This is because the eventual breakdown of lipid peroxides produces the low molecular weight volatiles which impart off-odor. Trace amounts of lipid peroxides have been detected using ultrasensitive techniques in the living animal (Miyazawa et al., 1996; Thomas et al., 1994). In just-killed fish, the levels of lipid peroxides were higher in physiologically active tissues such as liver and dark muscle compared to ordinary muscle (Nakamura et al., 1998). To work under conditions closer to the physiological status of lipids at the time of death, sodium borohydride was used to reduce the preformed lipid peroxide content of linoleic acid in the experiments of this study. At a lipid peroxide level of 3.4 μM, methemoglobin caused negligible peroxidation of linoleic acid, but reduced hemoglobins caused large gains in lipid peroxides under the same conditions (Table 2). These data suggest a possible role for reduced hemoglobins in the increase of lipid peroxides in the early stages of lipid oxidation.

Reduced hemoglobins can be more pro-oxidative than methemoglobin for a number of reasons. First, reduced hemoglobins can autoxidize, whereas methemoglobins cannot. Superoxide anion radical formed from oxyhemoglobin autoxidation can dismutate to hydrogen peroxide. The hydrogen peroxide formed can activate the methemoglobin which forms along with superoxide anion radical from oxyhemoglobin autoxidation (Kanner et al., 1987; Misra and Fridovich, 1972). If methemoglobin is the initial reactant, then there is no source of heme-bound oxygen that can be released as superoxide anion radical and reduced to hydrogen peroxide to activate the heme protein. Reduced myoglobins were shown to have higher pro-oxidative activity than metmyoglobins when exposed to liposomes, which was attributed to oxymyoglobin autoxidation (Chan et al., 1997). Second, reduced hemoglobins but not methemoglobins can act as Fenton reagents, producing the hydroxyl radical (Puppo and Halliwell, 1988). Third, alkoxyl radicals are formed in reduced hemoglobin systems exposed to *tert*-butyl hydroperoxide, while hemichromes are formed in methemoglobin systems (Thornalley et al., 1983). Alkoxyl radicals are capable of initiating lipid peroxidation due to their high redox potential (Buettner, 1993). Hemichromes are not considered as catalysts of lipid peroxidation (Baron et al., 2000).

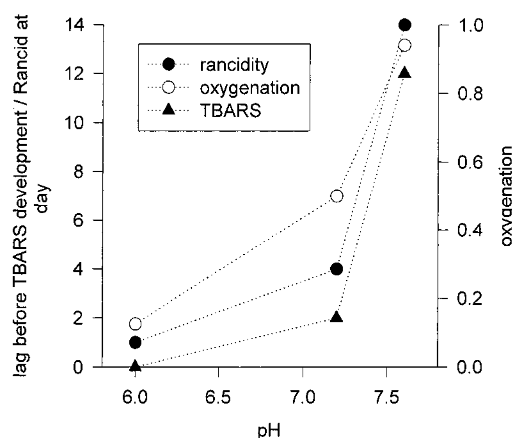

Figure 4. Relationship between oxygenation of trout hemoglobin component IV and rancidity development in washed cod with added trout hemolysate as a function of pH. Oxygenation values were taken from Binotti et al. (1971). A comparison between oxygenation and lag time before TBARS development is also presented.

The term “reduced hemoglobins” is used because reduced hemoglobins are comprised of a mixture of oxyhemoglobin and deoxyhemoglobin. The relative amounts of oxy- and deoxyhemoglobin vary as pH is adjusted. In the range of pH 6.0−8.0, component IV of trout hemoglobin exhibited a wide range of oxygen affinity (Binotti et al., 1971). Four distinct types of trout hemoglobin were isolated by electrophoresis, and component IV made up 65% of the total hemoglobin. Component IV is classified as an acidic hemoglobin on the basis of its electrophoretic mobility and its ability to bind oxygen being dependent on pH. Components I and II are basic hemoglobins whose oxygen binding is independent of pH (Zolese et al., 1999). We wished to compare the percentages of deoxyhemoglobin in component IV at pH 6.0, 7.2, and 7.6 determined by Binotti (1971) to the development of rancidity when hemolysate was added to washed cod at those pH values. The percentages of deoxyhemoglobin were closely related to our observation on rancidity development in this study (Figure 4). The lag phase before TBARS development also appeared to be related to the percentages of deoxyhemoglobin at the respective pH values (Figure

4). Therefore, a potential for deoxyhemoglobin to act as a catalyst of lipid oxidation is suggested.

Deoxyhemoglobin may become a catalyst of lipid oxidation on the basis of conformational changes of the protein that occur upon deoxygenation. A change in quaternary structure of the protein occurs with oxygen release (Stryer, 1988). Deoxyhemoglobin is less compact than oxyhemoglobin due to the change in quaternary structure. This could allow better access of fatty acids to the heme crevice. Rao et al. (1994) demonstrated that myoglobin that was mutated to increase access of linoleic acid to the heme crevice caused greater peroxidation of a fatty acid compared to nonmutated myoglobin. Further, greater heme pocket flexibility has been reported for partially oxygenated hemoglobins compared to fully oxygenated hemoglobins (Levy and Rifkind, 1985). If the catalysis of lipid oxidation is through the breakdown of trace amounts of preformed lipid peroxides, then the reaction of heme with a lipid peroxide should be sterically controlled as well. Deoxygenation moves the iron atom of the heme out of the plane of the porphyrin (Stryer, 1988). The exposure of the iron may be necessary to allow lipid peroxides to react in the heme crevice. Using numerical integration techniques, the rate of lipid peroxidation of egg liposomes by oxy-, met-, and deoxyhemoglobin were evaluated (Pietrzak and Miller, 1989). Deoxyhemoglobin was found to be around 3.5× more peroxidative than oxy- and methemoglobin on the basis of oxygen uptake and methemoglobin formation.

Ever et al. (1976) showed that the rate of hemoglobin oxidation by hydrogen peroxide was highest under nitrogen and decreased with increasing oxygen pressure. This suggests that deoxyhemoglobin reacts with hydrogen peroxide more readily than oxyhemoglobin. Paganga et al. (1992) showed a sudden, nearly complete conversion of oxyhemoglobin to deoxyhemoglobin at about the same time as lipoprotein oxidation exponentially increased. Ferryl hemoglobin formation also began to increase at around the same time as the deoxygenation occurred. These authors suggested the ferryl form of hemoglobin was the active catalyst, but the instantaneous conversion from oxyhemoglobin to deoxyhemoglobin suggests that the deoxygenated form of hemoglobin may be involved.

The model system consisting of trout hemolysate added to washed cod was used for the following reasons. Hemolysate, which is comprised of the soluble components of erythrocytes, was used as a source of hemoglobin. Hemoglobin is the major component in the hemolysate and the most probable catalyst considering its high concentration relative to that of any other catalyst present. Roughly one-third of the wet weight of packed erythrocytes is hemoglobin (Pennell, 1974). Hemolysate from trout was used since it is one of the few species that could be kept alive in our facilities. Live trout were needed to have a convenient source of freshly prepared hemolysate. In addition, the hemoglobin of trout is well characterized compared to other food fishes. Since it is generally thought that it is the membrane lipids that oxidize first (Gandemer, 1999), we wanted to develop a model system that would evaluate the response to this sensitive lipid fraction. Cod muscle was used as the lipid substrate since essentially all of its lipid is associated with its membranes. Trout has flesh with a moderate degree of lipid content. Thus, there could be some contamination with fish oil in the sample. Further, the mitochondria content is low in cod muscle compared to trout muscle. Mitochondria is a source of reactive oxygen species that could confound lipid oxidation due to added hemolysate. Washing cod muscle removes aqueous antioxidants and pro-oxidants from the muscle, while insoluble proteins and membrane phospholipids remain. Pro-oxidants and antioxidants are removed so to not interfere with reactions caused by added hemolysate. Removal of pro-oxidants from muscle tissue by washing is most effective when endogenous levels of pro-oxidants such as heme proteins and metals are low. Since cod muscle has low levels of pro-oxidants, washed cod muscle was used as a source of oxidizable lipid.

In the linoleic acid model system, increasing the preformed lipid peroxide level from 3.4 to 7.9 μM activated methemoglobin as a catalyst of lipid oxidation (Table 2). The level of preformed lipid peroxides in the washed cod was around 13 μM (data not shown). This high level of preformed lipid peroxides in the washed cod may have been part of the reason methemoglobin promoted rapid and extensive oxidation of washed cod lipids. Baron et al. (2000) showed that when metmyoglobin was exposed to linoleic acid emulsions (lipid hydroperoxide content <0.5 μM) containing H_2O_2 (12 μM), competitive formation of hemichrome and perferryl myoglobin radical occurred, favoring hemichrome, which resulted in little lipid peroxidation. It is possible that increasing the lipid hydroperoxide level causes a shift favoring perferryl radical formation, which is capable of initiating lipid peroxidation (Kanner et al., 1987). Oxy/deoxyhemoglobin was active at both levels of preformed lipid peroxides in the linoleic acid substate (Table 2). This suggests some factor other than preformed lipid peroxides controls the catalytic activity of reduced hemoglobins. The highly inhibitory effect of pH 7.6 on lipid oxidation when reduced hemoglobins were added to washed cod may be because the ratio of deoxyhemoglobin to oxyhemoglobin was below the threshold required to catalyze lipid oxidation. Greatly increasing the ratio of deoxyhemoglobin to oxyhemoglobin can be achieved by lowering the pH. When the pH was lowered from 7.6 to 6.0, lipid oxidation of washed cod by reduced hemoglobins rapidly occurred. Methemoglobin was as active a catalyst as reduced hemoglobins at pH 6.0, but methemoglobin retained its activity at pH 7.6 in the washed cod model system. Thus, it appears in these studies that methemoglobin catalyzed lipid oxidation by a lipid hydroperoxide-dependent mechanism while reduced hemoglobins operated via a pH-dependent mechanism.

Acidity enhancing the release of oxygen from hemoglobin without a change in valence state is termed the Bohr effect. One difference between hemoglobin and myoglobin is that myoglobin does not exhibit the Bohr effect (Stryer, 1988). Lipid oxidation of liposomes by myoglobin occurred more rapidly as the pH was reduced from 7.2 to 5.6 (Yin and Faustman, 1993). If a Bohr effect does not occur in myoglobin, oxygenation fluctuation will not occur with pH change, and hence a Bohr effect cannot be used to explain their result. Increased autoxidation of myoglobin at reduced pH (Chow et al., 1987; Shikama and Matsuoka, 1986) was used by Yin and Faustman (1993) to explain the fact that myoglobin caused greater lipid oxidation in the liposomes at

reduced pH. Hemoglobin autoxidation occurred more readily in our hemolysate solutions at pH 6.0 compared to pH 7.2 (Figure 3). The more rapid rate of oxyhemoglobin autoxidation at reduced pH may have contributed to the more rapid hemolysate-induced oxidation of washed cod lipids at reduced pH (Figure 1).

Another way that deoxyhemoglobin can affect lipid oxidation reactions is through its ability to increase the autoxidation rate of oxyhemoglobin (Rifkind et al., 1987). At pH 7.4, the autoxidation rate of human hemoglobin was optimal at around 55% oxygenation (Balagopalakrishna et al., 1996). It would be of interest to determine the relationship between deoxyhemoglobin content and the autoxidation rate at various pH values.

Adenosine triphosphate and ADP increased the deoxygenation of trout hemoglobin (Table 1). This could help explain the results of Undeland et al. (1999), who showed that extracts from very fresh herring were more pro-oxidative than extracts from older fish added to linoleic acid. ATP and ADP are quickly metabolized post mortem. These phosphated nucleotides may be present in extracts from freshly killed fish but not stored fish. Increased deoxygenation of hemoglobin caused by ATP could bolster the pro-oxidative activity of the heme protein.

A calculation was done to estimate how mincing muscle from just-killed fish could affect the amount of ATP available to hemoglobin. The concentrations of ATP in whole blood and fresh muscle are around 0.4 and 10 mM, respectively (Pennell, 1974; Hultin, 1985). Assuming a 1% blood volume in muscle, the ATP concentration available to hemoglobin would increase from 0.4 mM in the capillaries to 9.9 mM in thoroughly minced muscle. Our results suggest this influx of ATP could reduce the oxygenation of hemoglobin and consequently stimulate lipid oxidation (Table 1).

In conclusion, lowering the pH from 7.2 to 6.0 reduced the oxygenation of hemoglobin, enhanced the autoxidation rate of hemoglobin, and accelerated hemolysate-mediated lipid oxidation of washed cod muscle. Reduced hemoglobins but not methemoglobin stimulated lipid peroxidation when the level of preformed lipid peroxides was 3.4 μM. Future work should examine components or factors that modulate the oxygenation or deoxygenation of heme proteins and the consequent effect on lipid oxidation. Further, more research is needed to determine the link between autoxidation of hemoglobin and rancidity development.

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Received for review September 27, 1999. Revised manuscript received May 3, 2000. Accepted May 11, 2000. This work was supported in part by the Massachusetts Agricultural Experiment Station, by Grant No. 97-35503-4531 of the USDA National Research Inititiative Competitive Grants Program, and by Project Number 5700000741 of the MIT Sea Grant College Program.

JF991059W