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Effects of Fish Heme Protein Structure and Lipid Substrate Composition on Hemoglobin-Mediated Lipid Oxidation

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Hemoglobin (Hb) promoted lipid oxidation more effectively in washed tilapia as compared to washed cod in spite of a 2.8-fold higher polyenoic index in the washed cod. This suggested that increasing the fatty acid unsaturation of the substrate did not accelerate the onset of lipid oxidation. Substantial phospholipid hydrolysis in the washed cod was observed, which has the potential to inhibit lipid oxidation. MetHb formation and lipid oxidation occurred more rapidly at pH 6.3 as compared to pH 7.4. Trout Hb autoxidized faster and was a better promoter of lipid oxidation as compared to tilapia Hb. The greater ability of trout Hb to promote lipid oxidation was attributed in part to its lower conformational and structural stability based on secondary and tertiary structure, acid-induced unfolding, and thermal aggregation measurements. It is suggested that the structural instability and lipid oxidation capacity of trout Hb were at least partly due to low hemin affinity. Trout and tilapia Hb were equivalent in their ability to cause lipid oxidation in washed cod muscle heated to 80 °C. Apparently, these high temperatures denature both trout and tilapia Hb to such an extent that any differences in conformational stability observed at lower temperatures were negated.

KEYWORDS: Secondary structure; protein unfolding; lipid oxidation; heme proteins; hemin affinity; fatty acid composition; lipase

INTRODUCTION

Lipid oxidation is a major cause of quality deterioration in muscle foods manifested by formation of off-odors and off-flavors (*1*). Hemoglobin (Hb) is an effective promoter of lipid oxidation (*2*). This is relevant in muscle tissues used for food because substantial quantities of blood and hence Hb remain in the musculature even after bleeding (*3*, *4*). The only detectable heme pigment in chicken breast muscle from bled birds is Hb (*5*). Lipid oxidation in minced, trout muscle is attributed to dispersion of Hb from the tissue disruption (*6*).

Hb is made up of four polypeptide chains with each chain containing one heme group, the latter containing an iron atom coordinated inside the heme ring. Myoglobin (Mb) is a monomeric heme protein within the muscle cell with properties similar to Hb. Mb is more prevalent in dark muscles as compared to light muscle tissues (*5*). A better understanding of the mechanisms by which Hb and Mb promote lipid oxidation could lead to improved strategies to inhibit lipid oxidation during refrigerated and frozen storage.

Numerous studies have shown that certain Hbs promote lipid oxidation more effectively as compared to Hbs from other species. For example, herring and mackerel Hbs oxidized washed cod lipids more effectively as compared to trout Hbs (*7*). Trout Hb promoted lipid oxidation more effectively as compared to bovine Hb, while avian Hbs had intermediate activity (*8*). Oxygen affinity appeared to play a role in that those Hbs with elevated deoxyHb contents at pH 6.3 (e.g., trout Hb) promoted lipid oxidation most effectively at pH 6.3. Hb from pollock, mackerel, menhaden, and flounder were all found to be equally as pro-oxidative at pH 6, while they differed significantly in their ability to oxidize cod membrane lipids at pH 7.2 (*9*). The higher activities at pH 6 could be explained by higher and more rapid formation of deoxy and metHb, while differences between the Hbs at pH 7.2 could not be explained by these. It was hypothesized that structural differences between the four Hbs could have contributed to the differences seen at pH 7.2. Among yellow fin tuna Mb, Pacific green sea turtle Mb, and sperm whale Mb, yellow fin Mb had the most rapid oxygen dissociation rate and the highest susceptibility to

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autoxidation (*10*). The three Mbs had similar heme contact residues but varied in residues that were involved in globin stabilization and in postulated ligand paths to the heme iron. Hb and Mb autoxidation is the conversion of ferrous heme proteins (oxyHb and deoxyHb) to their oxidized forms (e.g., metHb). Heme is anchored more loosely in metHb as compared to ferrous Hb (*11*). Mb mutants with low heme affinity promoted lipid oxidation in washed cod more effectively as compared to mutants with high heme affinity (*12*).

Autoxidation of Hb from cold-adapted fish was found to be 10-fold faster as compared to warm-water fish at all temperatures tested, while high hydrostatic pressure did not affect the autoxidation rate (*13*). The rate of autoxidation was lower in monomeric Hbs (e.g., hagfish and lamprey) than in tetrameric Hbs from carp, tuna, and pig (*14*). It was also found that glutamine substitution at the E7 position did not accelerate autoxidation and that pig Hb oxidized slower than fish Hb. Autoxidation of sardine, sperm whale, saury, bonito, carp, and sheep Mb was studied as a function of temperature after heating and cooling (*15*). These authors found that fish Hbs had higher susceptibilities to autoxidation as compared to mammalian Hbs, which were associated with looseness in the protein structure due to heme pocket perturbations that resulted from thermal modulations.

Previous studies mostly focus on either heme protein structure or lipid oxidation capacity of different Hbs. The objective of this work was to examine protein structure and lipid oxidation capacity using Hb from warm-water fish as compared to cold-water fish. Furthermore, lipid substrates from warm and cold water fish were examined.

MATERIALS AND METHODS

Chemicals. Cumene hydroproxide, streptomycin sulfate, sodium heparin, ferrous sulfate, barium chloride, ammonium thiocyanate, tris-(hydroxylmethyl)aminomethane (Tris), guanidine hydrochloride (Gu-HCl), urea, superoxide dismutase, catalase, and sodium phosphate (Monobasic) were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were analytical grade and diluted in deionized water.

Blood Collection. Rainbow trout (*Onchorhynchus mykiss*) (25−40 cm) were maintained in tanks at the University of Wisconsin, Water Science and Engineering Laboratory (Madison, WI). Tilapia (*Oreochromis niloticus*) was obtained from Evans Farm (Pierson, FL) and maintained in tanks at the University of Florida, Department of Food Science and Human Nutrition. Four parts of blood was added to one part of 150 mM NaCl and sodium heparin (120 units/mL). The trout were bled from the caudal vein according to Rowley (*16*) using aminobenzoic acid ethyl ester as an anesthetic (0.5 g/L swimming water). Tilapia was placed on ice (pectoral side facing up) for 1 min, and blood was drawn via the caudal vein. Hbs were prepared within 24 h of blood collection.

Preparation of Hbs. 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 a Beckman J-6B centrifuge (Beckman Instruments Inc., Palo Alto, CA). After the plasma was removed, the red blood cells were washed by suspending them three times in 10 volumes of the above buffer (*17*). Cells were lysed in 3 volumes of 1 mM Tris, pH 8.0, for 1 h. One-tenth volume of 1 M NaCl was added to aid in stromal removal before ultracentrifugation (28000*g* for 15 min at 4 °C) using Beckman L8-70M ultracentrifuge (Beckman Instruments Inc.). Hemoylsates were then passed through DG-10 gel filtration columns (Bio-Rad, Hercules, CA). Hb solutions were stored at −80 °C or in dry ice prior to use.

Quantifying Hb Levels. The method of Brown (*18*) was adapted. Concentrated Hb solutions were diluted with 50 mM Tris, pH 8.0, buffer. Around 1 mg of sodium dithionite was added to 1.5 mL of the Hb solution and mixed in a cuvette. Carbon monoxide gas (Badger Welding, Madison, WI) was then bubbled into the samples for 30 s. The sample were scanned from 440 to 400 nm (Soret band) against a blank that contained only buffer using a double-beam spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, MD). The peak at 420 nm was recorded.

Preparation of Washed, Minced Fish Muscle. Cod (*Gadus morhua*) and tilapia (*O. niloticus*) fillets without skins were used. All dark muscle was removed. Cod light muscle (around 1 kg) was ground in a KS M90 mincer (Kitchen Aid Inc., St. Joseph, MI), and the tilapia was ground in an Oster heavy-duty food grinder (Sunbeam Products, Inc., Boca Raton, FL). The mince was then 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. The mixture was then allowed to stand for 15 min before dewatering with a fiberglass screen. Mince was then mixed with 50 mM sodium phosphate buffer (pH 6.3) at the same 1:3 ratio and homogenized (setting 1) using a Polytron Type PT 10/35 (Brinkmann Instruments, Westbury, NY) for the washed cod and using an Ultra Turrax T18 (IKA Works, Wilmington, NC) for the tilapia. It was allowed to stand for 15 min and finally centrifuged (15000*g* for 20 min at 4 °C). The resulting pellets (around 50 g each) were vacuum packaged (3 mil barrier), frozen at −80 °C, and subsequently used as the washed cod and washed tilapia muscle.

Addition of Hbs to Washed Fish Muscle. An appropriate volume of the Hb stock was added on a Hb basis to a final concentration of 12 μmol per kg washed cod and then stirred with a plastic spatula for 3 min to distribute the heme protein. Streptomycin sulfate (220 ppm) was added to inhibit microbial growth during storage. The pH values of the samples were checked just after addition of Hb, periodically during storage, and finally at the end of storage. To measure the pH, about 0.5 g of sample was diluted in 10 volumes of distilled, deionized water and homogenized, and the readings were recorded using an Accumet AR50 pH meter (Fisher Scientific, Pittsburgh, PA) or a model 220 Denver Instrument pH meter (Denver Instruments, Denver, CO). The final moisture content of the samples stored at 2 °C was adjusted to 88%. The pH was adjusted by addition of 1 M NaOH or 1 M HCl to reach the pH of 6.3 or 7.4. For the cooked study, the samples were prepared as mentioned above and then placed in a 80 °C water bath until the internal temperature reached 80 °C. The sample was then placed on ice, and the first sample was taken when the internal temperature was approximately 4 °C.

Relative Oxygenation of Trout and Tilapia Hb. Solutions containing Hb were scanned from 630 to 500 nm using a double-beam spectrophotometer model UV-4201 (PC) (Shimadzu Instruments, Inc.). The blank contained only buffer. The absorbance at the peak (575 nm) minus the absorbance at the valley (560 nm) was calculated. Larger differences indicate that the Hb was more highly oxygenated (*19*).

Measuring MetHb Formation. Hb solutions were diluted into 20 mM sodium phosphate buffer (pH 6.3 or 7.4). Spectra were obtained at regular time intervals between 700 and 500 nm using the UV-2401 spectrophotometer. The percentage of metHb was calculated according to the equations of Benesch et al. (*20*). For autoxidation studies, samples contained 3 mmol of superoxide dismutase and catalase per mol of heme to remove any superoxide and hydrogen peroxide that was produced during incubation. These reactive oxygen species stimulate formation of ferryl forms of Hb that confound measurement of metHb formation.

Circular Dichroism Spectroscopy. For determination of secondary structure and tertiary structure, Hb was placed in 2 (1.2 μM Hb) and 10 mm (12 μM Hb) quartz cuvettes (Wilmad Labglass, Buena, NJ), respectively, containing 20 mM sodium phosphate (monobasic) buffers at pH 6.3 and pH 7.4. The solution in the 2 mm cuvettes was scanned from 260 to 190 nm by an Aviv circular dichroism spectrometer (Aviv Biomedical, Inc., Lakewood, NJ) to determine secondary structure, while the solutions in the 10 mm cuvettes were scanned from 360 to 260 nm to determine tertiary structure. Resolution was set at 0.2 nm, bandwidth was set at 1 nm, sensitivity was set at 20 mdeg, response was set at 1 s, and scanning speed was set at 20 mdeg/min.

Gu-HCl-Induced Denaturation. Gu-HCl-induced denaturation of the two Hbs (trout and tilapia) was performed by incubating the protein (1.2 μM) in 0.0−3.0 M solutions of Gu-HCl (with 20 mM sodium phosphate) at pH 6.3 or pH 7.4 for an hour at 20 °C. At the end of the

incubation, 3 mL of each of the solutions was transferred to methacrylate cuvettes, and the cuvettes were placed in a HP 8453 diode array UV−visible spectrophotometer (Agilent Technologies, Palo Alto, CA). Single, full-spectrum (190−1100 nm) scans were run on each solution, with the extent of denaturation monitored by the change in heme peak absorbance at 414 nm. The fraction of unfolded Hb was calculated according to a two-state unfolding mechanism (*21*):

$$f_{app} = \frac{f_U - f}{f_U - f_N}$$

where f_{app} = fraction of unfolded Hb, f = observed maximum heme peak absorbance for Hb under the given conditions, f_N = observed maximum heme peak absorbance for a fully native Hb (at 0 M Gu-HCl), and f_U = observed maximum heme peak absorbance for a fully unfolded Hb (at 3 M Gu-HCl).

Isothermal Denaturation. Hb (1.2 μM) was added to quartz cuvettes containing 3 mL of preheated (50 °C) 20 mM sodium phosphate buffer solutions at either pH 6.3 or pH 7.4. The solution was immediately stirred, and cuvettes were sealed with Parafilm and a plastic cuvette lid to prevent evaporation. The cuvette was placed in a thermally controlled cuvette holder, and the solution was kept at a constant 50 °C with the aid of a Neslab RTE 7 computer-controlled water bath (Thermo Electron Corp., Newington, NH). Kinetic scans (350−800 nm) were run every 10 s for 1 h with a HP 8453 diode array UV−visible spectrophotometer (Agilent Technologies). Thermal aggregation was assessed by changes in absorbance of the protein at 750 nm, and percent protein aggregation was calculated according to the following formula:

$$\% \text{ protein aggregation} = (A_S - A_t/A_S - A_A) \times 100$$

where A_S = absorbance at 750 nm of soluble native Hb, A_t = absorbance at 750 nm of Hb at a given time point during isothermal treatment at 50 °C, and A_A = absorbance at 750 nm of denatured fully aggregated Hb.

Differential Scanning Calorimetry (DSC). Hb (12 μM) was added to a 20 mM sodium phosphate (monobasic) buffer at pH 7.4. The sample was transferred to a sample cell inside an ultrasensitive microdifferential scanning calorimeter (MicroCal LLC, Northampton, MA). The reference cell was filled with 20 mM sodium phosphate (monobasic) buffer at pH 7.4, with no protein added. The samples were then run from 20 to 90 °C at 1.5 °C/min, and the change in specific heat capacity of the Hb was recorded as a function of temperature. The temperature where a maximum endothermic peak was observed was defined as the thermal transition temperature.

Determination of Thiobarbituric Acid Reactive Substances (TBARS). TBARS were determined according to a modified procedure of Buege and Aust (*22*). On the day of use, 50% trichloroacetic acid containing 1.3% TBA was heated to 65 °C to dissolve the TBA. The sample was added to the TCA−TBA mixture (1:10) (w:v) and incubated for 1 h at 65 °C. After the mixture was centrifuged (2500*g* for 10 min), the absorbance of the supernatant at 532 nm was determined.

Determination of Lipid Peroxides. Between 0.4 and 0.5 g of washed cod and tilapia muscle was homogenized in 5 mL of chloroform/methanol (1:1) for 30 s using a Polytron Type PT 10/35 (Brinkmann Instruments) or an Ultra Turrax T18 (IKA Works, Wilmington, NC). Subsequently, the homogenizer was rinsed for 30 s with 5 mL of solvent. The homogenate and wash solution were then combined. Three milliliters of 0.5% NaCl was added, and the mixture was mixed for 30 s with a Vortex before centrifugation for 10 min (4 °C and 700*g*) to separate the mixture into two phases. Then, 1.33 mL of ice-cold chloroform/methanol (1:1) was added to 2 mL of the lower phase and mixed briefly. Twenty-five microliters of ammonium thiocyanate (4.38 M) and 25 μL of iron(II) chloride (18 mM) was added to the assay for lipid peroxides, and the samples were incubated for 20 min at room temperature before the absorbance at 500 nm was determined (*23*). The chloroform used contained ethanol as a preservative to eliminate high blank reading. Cumene hydroperoxide was used as a standard.

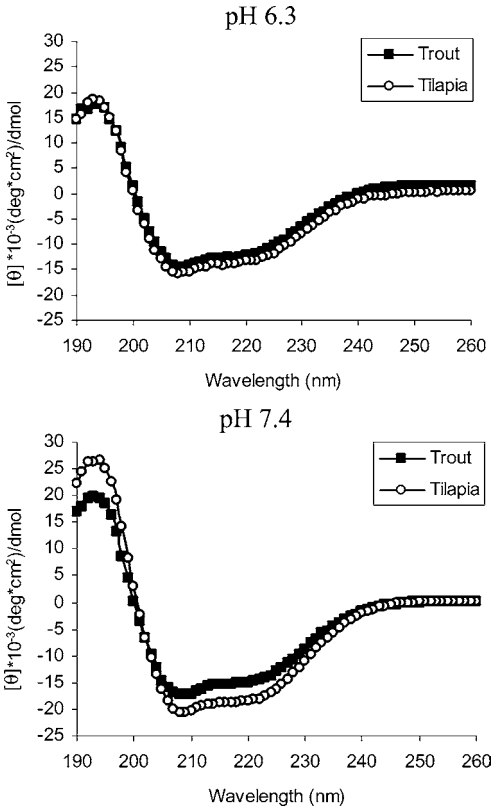

Figure 1. Near-UV circular dichroism spectra of trout and tilapia Hb at pH 6.3 and 7.4. The protein concentration was 1.2 μM, and spectra were recorded at 5 °C.

Sensory Analysis. Three to four trained panelists sniffed the headspace above the samples that were held in 30 mL amber bottles during ice storage to detect the onset and development of painty odor. A break time of approximately 5 min between analyses by each panelist was employed to allow for equilibration of volatiles between the washed cod and the headspace. A scale of 0−10 with 10 being the strongest was utilized. Slightly rancid references were provided by blending heavily oxidized menhaden oil with vegetable oil (mostly odorless) (*24*). This reference was used to train the panelist.

Cooking Procedure. Amber bottles (30 mL capacity) containing around 15 g washed cod and added Hb were placed in a waterbath held at 88 °C. Bottles were removed from the waterbath when the internal temperature of the tissue reached 80 °C. Bottles were then quickly transferred to an ice bath for rapid cooling prior to 2 °C storage (*25*).

Lipid Analysis of Washed Fish Muscle. Lipids were extracted using the Folch et al. method (*26*). Fatty acids were prepared according to the procedure of Maxwell and Marmer (*27*) using C23:0 as an internal standard. Fatty acid methyl esters were analyzed on a GC model 6850 (Agilent Technologies, Wilmington, DE) fitted with a DB-23 (60 m × 0.25 mm id., 0.25 μm film) capillary column (Agilent Technologies) as described by Bechtel and Oliveira (*28*). Data were collected and analyzed using the GC ChemStation program (Rev.A.08.03 [847]; Agilent Technologies 1990−2000). Hydrogen was used as a carrier gas at a constant flow of 1.0 mL/min with an average velocity of 30 mL/min. The initial nominal pressure of the inlet was 15.3 psi, and both injector and detector were held at a constant temperature of 275 °C. The split ratio was 25:1, and the oven programming was 140−200 °C at a rate of 2 °C/min, 200−220 °C at a rate of 1 °C/min, and 220−240 °C at a rate of 10 °C/min for a total run time of 52 min. The detector was operated at a constant makeup flow of 35 mL/min of nitrogen, with an air and hydrogen flow of 450 and 40 mL/min, respectively. An autosampler performed the GC injections of standards and sample, and the injection volume was 1 μL. The ChemStation enhanced integrator program was used to integrate the chromatogram peaks. All standards used in the identification of peaks were purchased

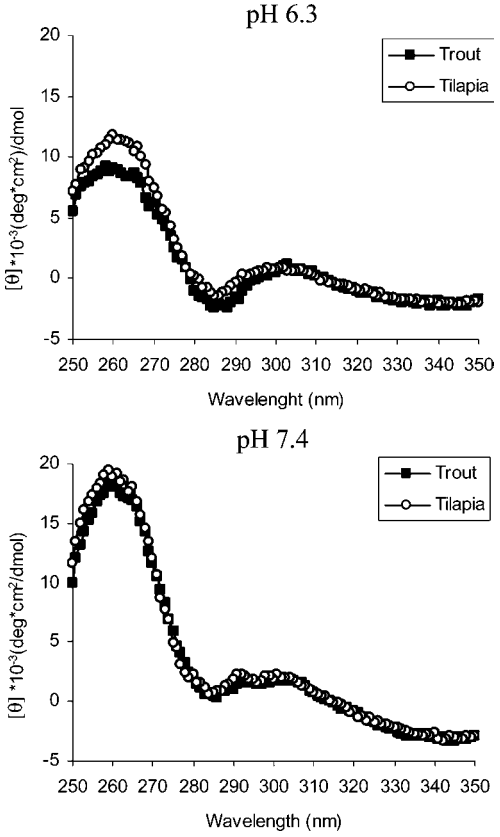

Figure 2. Far-UV circular dichroism spectra of trout and tilapia Hb at pH 6.3 and 7.4. The protein concentration was 12 μM, and spectra were recorded at 5 °C.

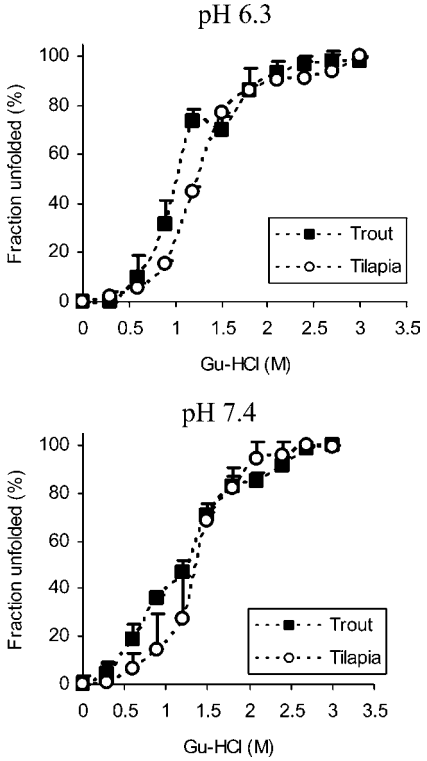

Figure 3. Gu-HCl-induced unfolding of trout and tilapia Hb at pH 6.3 and 7.4 as determined from the change in heme group absorbance (414 nm). The protein concentration was 1.2 μM, and the absorbance was read after 1 h at 20 °C.

from Supelco (Bellefonte, PA). The standards used were as follows: Supelco 37, Bacterial Acid Methyl Esters Mix, Marine Oil #1, and Marine Oil #3. Samples were run in duplicates, and cod liver oil was used as a secondary reference standard (*29*). A Iatroscan TLC/FID Analyzer model MK-6s (Iatron Laboratories Inc., Tokyo, Japan) was used to determine the distribution of the main lipid classes in the extracted lipids as described by Oliveira and Bechtel (*30*). Seven standards obtained from Sigma were used to identify the lipid classes and included cholesterol, tripalmitin, palmitic acid, L-α- phosphatidylcholine, 1,2-dipalmitoyl-*sn*-glycerol (1,2-DAG), 1,3-dipalmitoyl-*sn*-glycerol (1,3-DAG), and DL-α-monopalmitoylglycerol. The solvent system used was a mixture of hexane:ethyl ether:formic acid at the ratio of 80:25:1.2. In this system, 1,3-DAG coelutes with the sterol peak and is separated from the 1,2-DAG peak. Lipid classes were reported as percent triacylglycerides, 1,2-diacylglycerides, monoacylglycerides, free fatty acids, and the combined percentages of sterols and 1,3-diacylglicerides. The polyenoic index was calculated as follows: (% diene × 2 + % triene × 3 + % tetraene × 4 + % pentaene × 5 + % hexene × 6).

Statistical Evaluations. All experiments were done at least in duplicate, and in each experiment, Hbs from at least three different fish were separately evaluated. Analysis of variance with a MIXED procedure of the SAS system was used to evaluate data from storage studies (*31*). Means were separated by using differences of least squares.

RESULTS

Conformational and Structural Differences between Trout and Tilapia Hb. Several different methods were employed to obtain information on the structure, conformation, and stability of trout Hb as compared to tilapia Hb. Circular dichroism spectra were obtained for Hb at both pH 6.3 and pH 7.4 to determine if there were differences in their secondary (**Figure 1**) and tertiary (**Figure 2**) structures. The secondary structure spectra at pH 6.3 were similar for both Hb types, while spectra at pH 7.4 indicated that tilapia Hb had more secondary structure than trout Hb since molar ellepticity (θ) values were more negative at 222 nm (**Figure 1**). The spectra also showed that while trout Hb had similar spectra at both pH values, tilapia Hb had more secondary structure at pH 7.4 as compared to pH 6.3. Interestingly, the tertiary spectra (**Figure 2**) suggest that tilapia Hb has more tertiary structure at pH 6.3 than trout Hb, while there was almost no difference in the spectra at pH 7.4 between the two Hb. However, both Hb types had higher values of molar ellepticity (θ) from ~250 to 280 nm at pH 7.4 as compared to pH 6.3, suggesting a more tertiary structure at the higher pH.

Both Hb types were subjected to serial Gu-HCl-induced unfolding and changes in heme peak absorbance monitored to study the conformational stability of the two proteins (**Figure 3**). At pH 6.3, the onset of denaturation occurred at around 0.2 M Gu-HCl for both Hb types. Above 0.4 M Gu-HCl, the trout Hb exhibited more denaturation than tilapia Hb, and the midpoint of denaturation (50% unfolded) occurred at a ~0.2 M lower concentration of Gu-HCl for trout Hb. Values for both Hb types were similar above 1.5 M Gu-HCl. Both Hb types exhibited more stability toward Gu-HCl at pH 7.4 as compared to pH 6.3. Tilapia Hb also exhibited more stability than trout Hb at pH 7.4.

The two Hb types were also subjected to thermal denaturation experiments, by following protein aggregation at 50 °C (**Figure 4**). Tilapia Hb resisted thermal denaturation more at 50 °C as compared to trout Hb for both pH values tested. There was little difference in the thermal aggregation curve for trout Hb at both pH values, while tilapia Hb was clearly more stable toward thermal denaturation at pH 7.4. Similar to what was seen for the Gu-HCl-induced denaturation, the onset of thermal denaturation/aggregation occurred at a similar point for trout and tilapia Hb at pH 6.3, while it occurred at a later point for tilapia Hb at pH 7.4. To complement the thermal aggregation data,

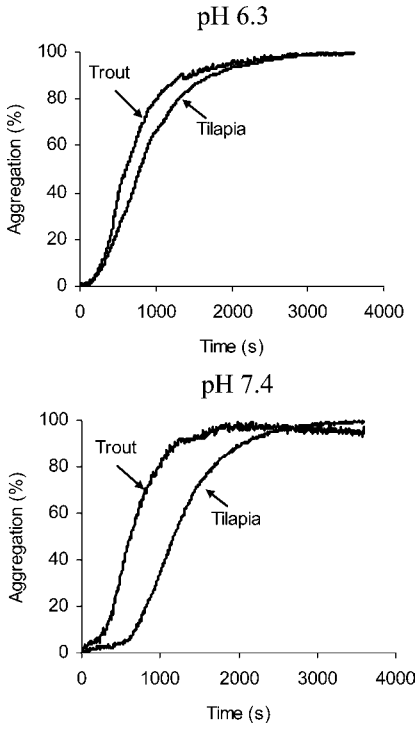

Figure 4. Isothermal denaturation of trout and tilapia Hb at pH 6.3 and 7.4 as determined by protein aggregation (absorbance at 750 nm). The protein concentration was 1.2 μM, and samples were heated at 50 °C for 1 h.

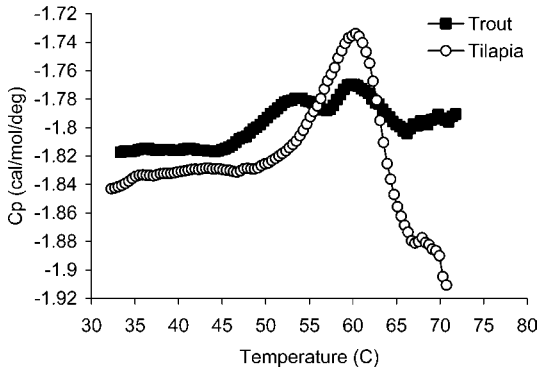

Figure 5. DSC scans of trout and tilapia Hb. The protein concentration was 12 μM, and solutions were at pH 7.4. Cp is indicative of heat capacity.

the two Hb samples were subject to micro-DSC analysis, which reveals the thermal stability of the two proteins (**Figure 5**). Scans were conducted only at pH 7.4, as reliable results could not be obtained at pH 6.3 due to extensive aggregation. The DSC scans revealed a single large peak for tilapia Hb with a midpoint at ~61 °C. Results for trout Hb suggest two smaller peaks at ~54 and ~60 °C.

Relative Oxygenation of Trout and Tilapia Hb. Hb oxygenation in trout and tilapia Hb was determined at pH 6.3 and 7.4. Hb oxygenation at pH 6.3 was lower in trout Hb as compared to tilapia Hb (**Table 1**). Both tilapia and trout Hb were highly oxygenated at pH 7.4. The oxyHb content in tilapia Hb was lower at pH 6.3 as compared to pH 7.4.

Met Hb Formation in Trout and Tilapia Hb. MetHb formation was measured in tilapia and trout Hb at pH 6.3 and 7.4 (**Figure 6**). Tilapia Hb was more resistant to metHb formation at pH 6.3 and pH 7.4 as compared to trout Hb ($p < 0.001$). MetHb formation occurred more rapidly at pH 6.3 as compared to pH 7.4 for both trout Hb ($p < 0.001$) and tilapia Hb ($p < 0.001$).

Table 1. Relative Hb Oxygenation of Trout and Tilapia Hb (20 μM) at pH 6.3 and 7.4[a]

	relative Hb oxygenation (peak, 575 nm; valley, 560 nm)	
	pH 6.3	pH 7.4
trout Hb	0.023 ± 0.003	0.140 ± 0.006
tilapia Hb	0.069 ± 0.009	0.133 ± 0.003

[a] Hb solutions were diluted in 20 mM sodium phosphate, pH 6.3 or 7.4. Hbs were in the ferrous oxidation state prior to taking measurements.

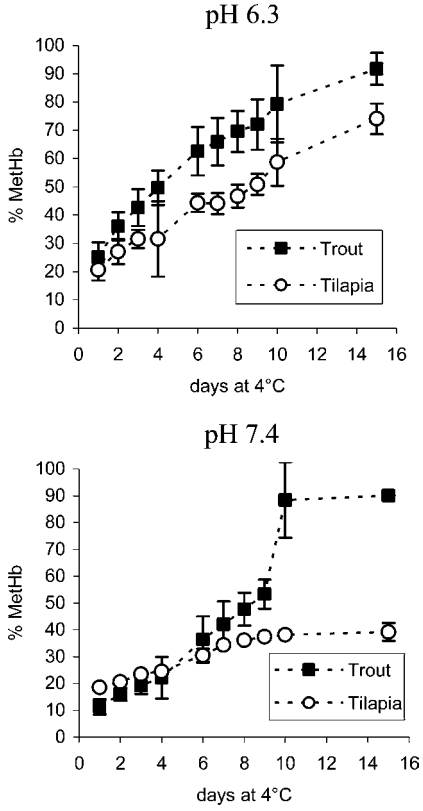

Figure 6. Formation of metHb from trout and tilapia (pH 6.3 and pH 7.4). The Hb concentration was 20 μM. Samples contained 3 mmol superoxide dismutase/catalase per mol of heme.

Lipid Oxidation Due to Trout and Tilapia Hb. Trout and tilapia Hb were separately added to washed cod muscle at pH 6.3 and 7.4 to assess the onset of lipid oxidation during storage at 2 °C. The formations of TBARS, painty odor, and lipid peroxides were used as indicators of lipid oxidation. At pH 6.3, TBARS values were higher at day two in trout Hb-containing samples as compared to those containing tilapia Hb ($p < 0.001$) (**Figure 7a**). By day 3, extensive TBARS formation was observed in both trout and tilapia Hb-containing samples at pH 6.3. At pH 7.4, TBARS did not increase until after day 8 of storage in trout Hb-containing samples (**Figure 7b**). TBARS remained low during the entire 14 day storage period in tilapia Hb-containing samples at pH 7.4. Although TBARS formation occurred more rapidly at pH 6.3 as compared to 7.4 in trout Hb samples, the maximal TBARS values were nearly equivalent at both pH values (**Figure 7a**,**b**).

Trout and tilapia Hb were also added to washed tilapia at pH 6.3 and 7.4. At pH 6.3, TBARS formation occurred rapidly and there was no difference in TBARS values when comparing trout Hb to tilapia Hb in washed tilapia (**Figure 7c**). TBARS

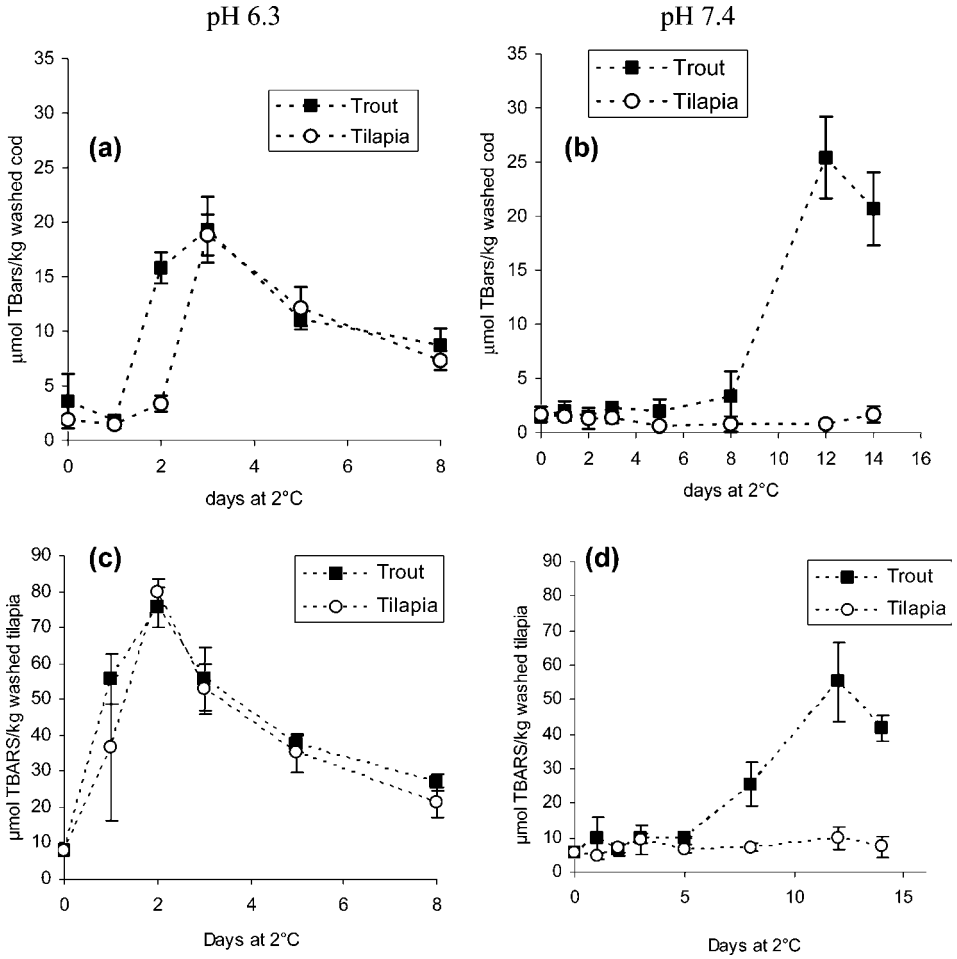

Figure 7. TBARS values obtained during 2 °C storage in washed cod (**a** and **b**) and washed tilapia (**c** and **d**) containing trout and tilapia Hb. The Hb concentration in each sample was 12 μmol/kg washed cod. The final pH values were pH 6.3 and 7.4.

formation was significantly suppressed at pH 7.4 as compared to pH 6.3 for both Hb samples (**Figure 7c**,**d**). At day 8, TBARS were higher in samples containing trout Hb as compared to tilapia Hb at pH 7.4. Each Hb promoted TBARS formation more effectively in washed tilapia as compared to washed cod at pH 6.3 (**Figure 7a**,**c**).

Painty odor development during 2 °C storage was assessed in washed cod containing added trout and tilapia Hb. A score of 1.5 indicated slight rancidity while a strongly rancid sample received a score of 10. At pH 6.3, painty odor developed by day 2 in trout Hb samples and by day 3 in tilapia Hb samples (**Figure 8**). Painty odor was not detected in samples containing tilapia Hb at pH 7.4 during the entire 14 day storage period, while trout Hb samples had moderate rancid odor at day 12 (**Figure 8**).

Lipid peroxides formed more rapidly in trout Hb samples as compared to tilapia Hb samples at pH 6.3 in washed cod based on values at day 2 ($p < 0.01$) (**Figure 9a**). At pH 7.4, low lipid peroxide values were obtained in trout and tilapia Hb-containing samples during 8 days of 2 °C storage (**Figure 9b**). Lipid peroxide formation was also followed in washed tilapia muscle containing trout and tilapia Hb. The formation of lipid peroxides at pH 6.3 was rapid in the washed tilapia similar to that observed for TBARS formation. Lipid peroxides did not form more rapidly from trout Hb as compared to tilapia Hb at pH 6.3 in washed tilapia (**Figure 9c**). Lipid peroxide formation was significantly slower and less at pH 7.4 for both Hb types (**Figure 9d**). Trout Hb led to more rapid and more extensive lipid peroxide formation at pH 7.4 as compared to tilapia Hb. Interestingly, each Hb promoted lipid peroxide formation more effectively and extensively in washed tilapia as compared to washed cod at pH 6.3 and 7.4 (**Figure 9a**−**d**).

The ability of trout and tilapia Hb to oxidize washed cod lipids after a cooking treatment was assessed at pH 6.3. After cooking to 80 °C, the samples were cooled on ice and stored at 2 °C for 3 days. There was no significant difference in TBARS values when comparing trout Hb- and tilapia Hb-containing samples during the storage period (**Figure 10**). Both trout Hb- and tilapia Hb-containing samples had elevated TBARS values after 20 h of storage. More variation in lipid peroxide values was observed in trout Hb-containing samples as compared to tilapia Hb samples during storage (**Figure 10**). However, there was no significant difference in lipid peroxide values when comparing trout Hb-containing samples to those containing tilapia Hb during storage.

Lipid Composition of Washed Cod and Washed Tilapia. The lipid content was higher in washed tilapia as compared to washed cod muscle (**Table 2**). The percentages of different lipid classes in washed cod and washed tilapia are shown in **Table 2**. The triacylglycerol contents were 41 and 1.2% in washed tilapia and washed cod, respectively. The phospholipid contents were 50.3 and 85.6% in washed tilapia and washed cod, respectively. The free fatty acid content was higher in washed cod muscle as compared to washed tilapia muscle (on a lipid basis).

Saturated, mono-, di-, tri-, tetra-, penta-, and hexaunsaturated fatty acid contents in washed cod and washed tilapia are shown in **Table 3**. The contents of highly unsaturated fatty acids (e.g.,

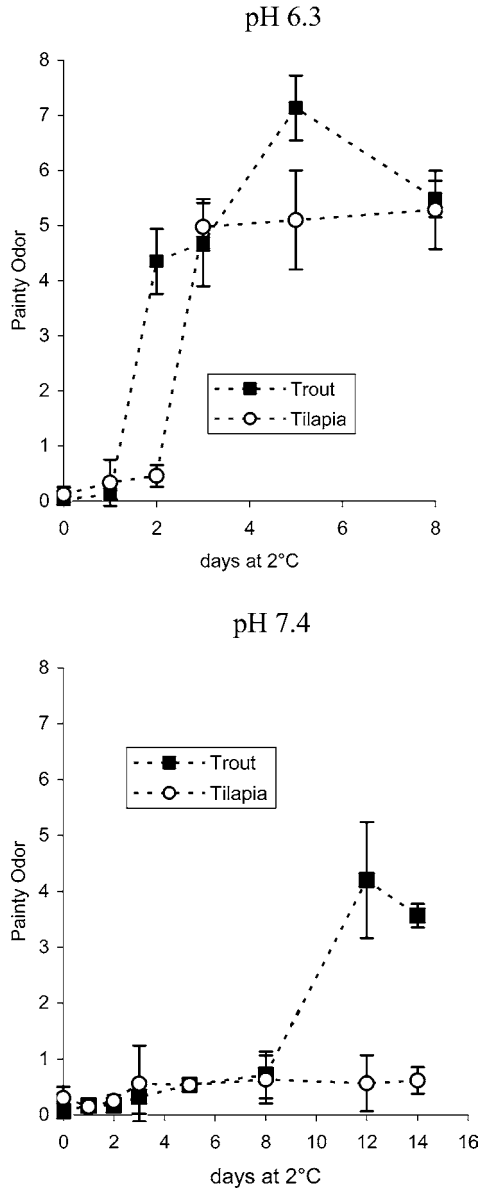

Figure 8. Sensory scores obtained during 2 °C storage in washed cod containing trout and tilapia Hb. The Hb concentration in each sample was 12 μmol/kg washed cod. The final pH values were 6.3 and 7.4. A score of 1.5 indicates slight painty odor.

fatty acids with five or six double bonds) were 12% in washed tilapia and 48% in washed cod. The total polyenoic index was 119 in washed tilapia and 329 in washed cod, indicating that the washed cod muscle contained around 2.8 times more polyunsaturation than washed tilapia. The complete fatty acid profiles for washed cod and washed tilapia are shown in **Table 4**.

DISCUSSION

Hb is a protein composed of four similar subunits, which are rich in α-helixes. Each subunit has a heme crevice containing a heme group, which has been shown to have a large influence on Hb structure and stability (*32*, *33*). Circular dichroism studies demonstrated that there was more secondary structure for both Hb types at pH 7.4 than 6.3, based on near-UV spectra (**Figure 1**). According to far-UV spectra (**Figure 2**), there was also substantially more tertiary and quaternary structures at pH 7.4 as compared to pH 6.3 for both proteins. Changes in Hb quaternary structure at the $\alpha 1\beta 2$ interface can be studied by changes in CD spectra in the 270−300 nm range resulting from aromatic residues (*34*, *35*). Within this region, changes at 285 nm are believed to report on the transition from ligated relaxed (R) to the unligated tense (T) Hb form (*36*). At pH 7.4, there were minor differences in the 270−300 nm range between both proteins, suggesting no tangible difference in their quaternary subunit interactions. Both trout and tilapia Hb showed a similar drop in ellipticity at pH 6.3 in the 270−300 nm range, which suggests a modified subunit interaction (*35*). A drop in ellipticity for both Hb at 285 nm as pH was reduced from 7.4 was indicative of a root effect, where a drop in pH leads to deoxygenation and a shift from the R form to the T form (*37*). This effect was larger for trout Hb than tilapia since its drop in ellipticity at 285 nm was larger. This is in agreement with our Hb oxygenation results (**Table 1**).

Changes in the L-band region around 260 nm report changes in the interactions between the globin and heme (*38*). Tilapia Hb had a higher ellipticity at 260 nm than trout Hb at pH 7.4, which suggests a stronger heme−globin interaction in the tilapia Hb. The large decrease in ellipticity at 260 nm for both proteins as pH was reduced to 6.3 is in agreement with previous studies and is believed to be due to weaker heme−globin interactions resulting from heme autoxidation as well as deoxygenation (*37*). This ellipticity change was larger for trout Hb than tilapia Hb, in agreement with our autoxidation and deoxygenation studies, which showed overall higher levels of autoxidation (**Figure 6**) and less oxygenation for trout Hb at pH 6.3 (**Table 1**). The heme group is a five coordinate high-spin complex with a histidine axial ligand (*39*) and is stabilized mainly by hydrophobic interactions from hydrophobic amino acids in the heme pocket as well as an iron-histidine proximal bond and H-bonding with a distal residue (*40*). As pH is reduced from 7.4 to 6.3, some of the more solvent-exposed histidine residues in Hb can become partly protonated, which could destabilize the native structure and lead to a conformational change in the heme pocket, which would explain the differences seen in both secondary and tertiary structures (*41*, *42*).

Smaller differences between pH values were seen in Gu-HCl-induced denaturation studies, while there were differences seen between the two types of Hb; tilapia Hb was more stable (**Figure 3**). Gu-HCl unfolds proteins by interacting more favorably with both polar and nonpolar amino acid residues than water (*43*). Gu-HCl will act on the whole protein molecule and not only the heme crevice (which becomes increasingly more unstable as pH is reduced), which could explain why the unfolding curve was little affected by pH since the heme environment alone was not being acted on. The slightly higher resistance of tilapia Hb to Gu-HCl-induced unfolding suggests that its overall protein structure, not only heme crevice, was more stable than trout Hb. Tang et al. (*11*) demonstrated that unfolding of deoxyMb was required for cleavage of the heme(II)iron−proximal histidine bond, whereas hemin loss from metMb was driven by protonation of the hemin(III) iron−proximal histidine bond, which will lead to instability of the globin. Thus, the oxidation state and ligand status of the heme protein should alter the mechanism by which heme protein structure is disrupted.

As stated before, CD data demonstrated that tilapia Hb had more secondary structure than trout Hb (**Figure 1**) and also suggests slightly stronger tertiary interactions (**Figure 2**), which can explain its higher resistance to Gu-HCl. Further evidence for higher stability of tilapia Hb comes from the isothermal aggregations studies (**Figure 4**). The increase in aggregation at 50 °C with time is due to increased denaturation of Hb. It is

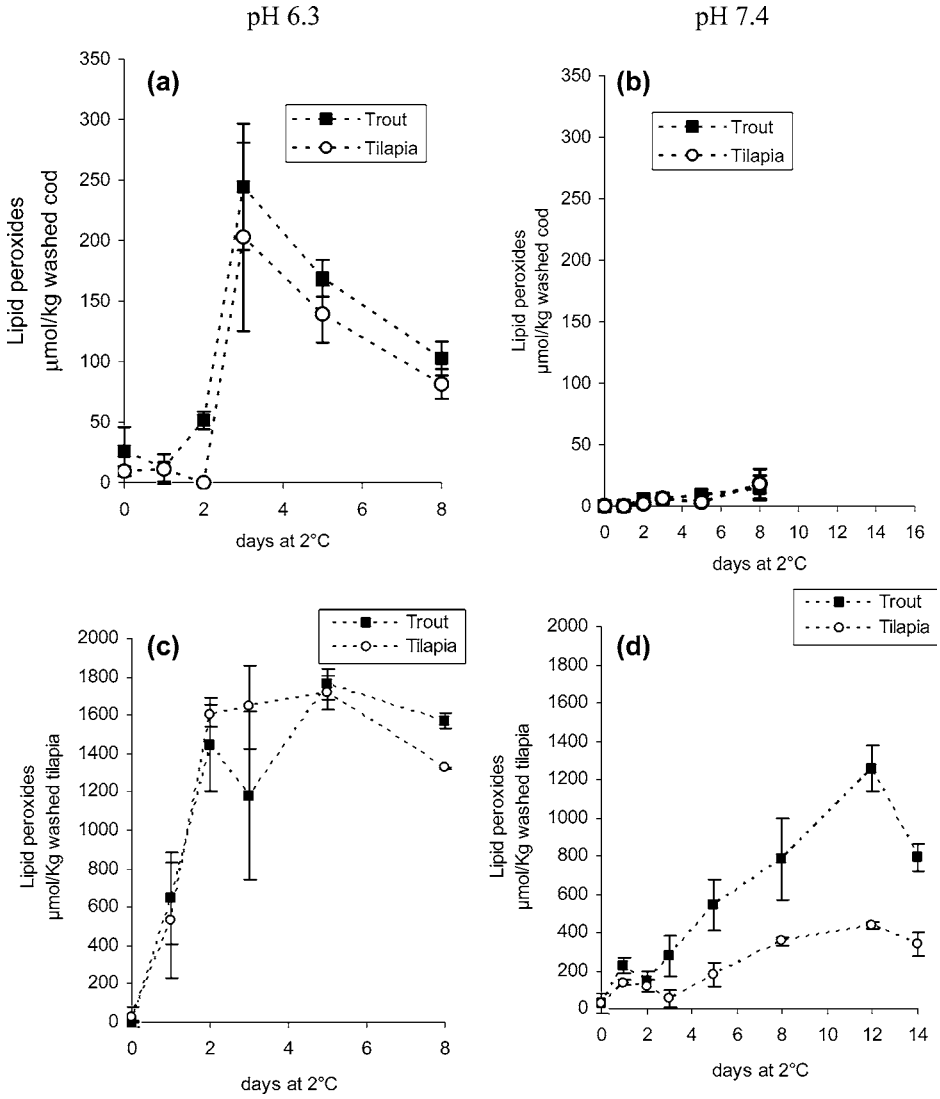

Figure 9. Lipid peroxide values obtained during 2 °C storage in washed cod (**a** and **b**) and washed tilapia (**c** and **d**) containing trout and tilapia Hb. The Hb concentration in each sample was 12 μmol/kg washed cod. The final pH values were 6.3 and 7.4.

interesting to note that there was very little difference in the onset or progression of isothermal aggregation at both pH values for trout Hb, while at pH 7.4 tilapia Hb was significantly more thermally stable as compared to pH 6.3. One likely reason for this difference is the heme crevice of these two proteins. The crevice and its heme are very hydrophobic and are thought to play a major role in Hb aggregation as degradation/unfolding of the heme peak has been found to precede protein aggregation (*44*, *45*). Studies comparing tilapia carboxy-Hb and oxy-Hb clearly demonstrated that if the heme group is stabilized with carbon monoxide, the protein becomes more thermally stable and is less prone to thermal aggregation (*45*). Because a lower pH destabilizes the heme crevice (i.e., heme and globin interaction), then based on these findings it was not unexpected that tilapia Hb was more stable at pH 7.4 than pH 6.3. Studies have also demonstrated that oxidation state of the protein plays an important role in its stability (*32*, *46*). Wittung-Stafshede (*32*) demonstrated with several heme proteins that the oxidized form is less thermally stable than the reduced form. Mony et al. (*46*) also demonstrated that oxidized tilapia Hb is substantially less stable than both oxy-Hb and carboxy-Hb. That study also demonstrated that CO-Hb was significantly more thermally stable than oxy-Hb, which was linked to its significantly greater resistance to autoxidation. Autoxidation studies performed at 4 °C demonstrated that trout Hb oxidized more readily than tilapia Hb (**Figure 6**), while they also showed that at pH 7.4 trout Hb was more stable to oxidation than at pH 6.3. However, the isothermal aggregation study was conducted at the 50 °C, and it is quite possible that at this high temperature any pH stabilizing effect of pH 7.4 would have been lost for trout Hb. Trout is a cold-water species, while tilapia is a warm-water species; thus, it is quite likely that this temperature had a lesser effect on tilapia Hb.

DSC studies demonstrated differences in tilapia thermal denaturation midpoints (**Figure 5**). Tilapia Hb had a single peak at 61 °C, while trout Hb had one peak at 52 °C and another at 60 °C. It is well-known that trout hemolysate has four distinct Hbs, which differ in their functions, conformation, and stability, with HbI and HbIV together making up about 80% of the four (*37*, *47*, *48*). The two different DSC peaks may reflect those two forms. While tilapia Hb had a peak at 60 °C, which may represent a more stable trout Hb form, the peak at 52 °C could have triggered the earlier aggregation seen for trout Hb at 50 °C and thus led to coaggregation of the other more stable forms in the trout hemolysate.

There are a number of explanations of why trout Hb promoted lipid oxidation more effectively than tilapia Hb in the raw state (**Figure 7**). The first involves oxygen affinity of the two heme

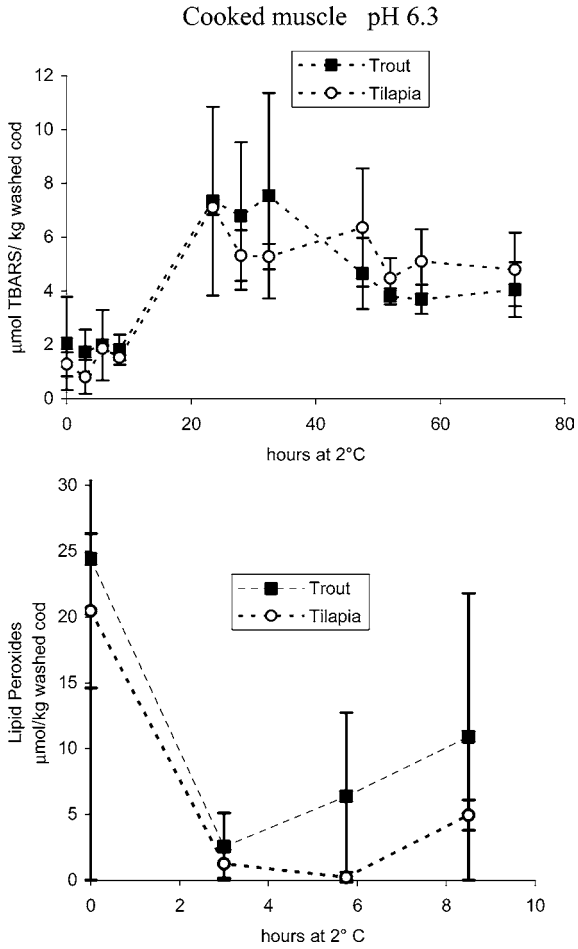

Figure 10. TBARS values and lipid peroxides obtained in washed cod containing trout and tilapia Hb that was heated to 80 °C prior to 2 °C storage. The Hb concentration in each sample was 12 μmol/kg washed cod.

Table 2. Percent Lipid in Washed Muscle (g/100 g Washed Muscle) and Percentage of Different Lipid Classes in Washed Cod and Washed Tilapia (g/100 g Lipid)[a]

lipid content and lipid classes	washed tilapia	washed cod
g/100 g washed muscle total lipid content	4.16 ± 0.27	0.67 ± 0.06
g/100 g lipid triacylglycerols	41.05 ± 1.09	1.19 ± 0.90
phospholipids	50.31 ± 0.38	85.62 ± 0.59
free fatty acids	2.32 ± 0.33	8.67 ± 2.57
1,2-diacylglycerols	ND	ND
sterols + 1,3-diacylglycerols	6.16 ± 1.14	4.49 ± 2.63
monoacylglycerols	0.33 ± 0.22	0.18 ± 0.02

[a] ND, not detected.

proteins. Trout Hb exhibited more of a root effect at pH 6.3 as compared to tilapia Hb (**Table 1**). The root effect is a decrease in oxygen affinity at reduced pH. Decreased oxygen affinity can accelerate lipid oxidation due to deoxyHb-mediated metHb formation as described previously (*49*) (reaction 1):

$$\text{deoxy}(+2)\text{Hb} + \text{H}_2\text{O} \rightleftarrows \text{deoxy}(+2)\text{Hb}\cdots\text{H}_2\text{O}$$

$$\text{deoxy}(+2)\text{Hb}\cdots\text{H}_2\text{O} + \text{O}_2 \rightarrow \text{met}(+3)\text{Hb} + \text{O}_2^{-\bullet} \quad \text{(reaction 1)}$$

MetHb formation did indeed occur more rapidly with trout Hb as compared to the more oxygenated tilapia Hb at pH 6.3

Table 3. Degrees of Fatty Acid Unsaturation in Washed Cod and Washed Tilapia (% w/w)

fatty acid	washed tilapia	washed cod
saturates	31.35 ± 0.60	22.65 ± 0.38
monounsaturates	33.84 ± 1.45	20.42 ± 1.97
diunsaturates	12.53 ± 0.51	1.02 ± 0.03
triunsaturates	4.71 ± 0.06	0.40 ± 0.01
tetraunsaturates	3.85 ± 0.18	5.18 ± 0.32
pentaunsaturates	6.53 ± 0.22	17.24 ± 0.40
hexaunsaturates	5.37 ± 0.31	30.65 ± 1.32
polyenoic index	119	329

Table 4. Fatty Acid Composition in Washed Tilapia and Washed Cod (% w/w)[a]

fatty acid	washed tilapia	washed cod
C12:0	0.07 ± 0.00	ND
C14:0	3.30 ± 0.11	0.62 ± 0.18
C15:0	0.52 ± 0.00	0.32 ± 0.05
C16:0	20.61 ± 0.54	18.59 ± 0.35
C17:0	0.69 ± 0.01	ND
C18:0	5.87 ± 0.03	3.11 ± 0.22
C20:0	0.29 ± 0.02	ND
14:1 ω 5	0.15 ± 0.01	ND
16:1 ω 13	0.19 ± 0.00	ND
16:1 ω 11	0.17 ± 0.01	0.31 ± 0.03
16:1 ω 9	0.63 ± 0.03	0.08 ± 0.14
16:1 ω 7	6.41 ± 0.21	1.93 ± 0.44
16:1 ω 5	0.33 ± 0.01	0.36 ± 0.04
17:1 ω 8	0.22 ± 0.07	ND
18:1 ω 9 *trans*	0.11 ± 0.00	1.71 ± 0.14
18:1 ω 9 *cis*	20.59 ± 1.20	7.91 ± 0.89
18:1 ω 7	3.44 ± 0.11	2.97 ± 0.29
18:1 ω 5	0.13 ± 0.01	0.33 ± 0.04
20:1 ω 11	1.08 ± 0.08	0.75 ± 0.10
20:1 ω 9	0.21 ± 0.13	1.93 ± 0.27
20:1 ω 7	0.17 ± 0.01	ND
22:1 ω 11	ND	1.13 ± 0.44
24:1 ω 9	ND	1.01 ± 0.26
16:2 ω 6	0.92 ± 0.05	ND
18:2 ω 6 *cis*	10.91 ± 0.54	1.02 ± 0.03
20:2 ω 6	0.71 ± 0.02	ND
18:3 ω 6	0.93 ± 0.04	ND
18:3 ω 4	0.11 ± 0.01	ND
18:3 ω 3	1.95 ± 0.04	0.40 ± 0.01
18:3 ω 1	0.69 ± 0.06	ND
20:3 ω 6	0.72 ± 0.01	ND
20:3 ω 3	0.32 ± 0.02	ND
18:4 ω 3	0.70 ± 0.04	0.71 ± 0.06
20:4 ω 6	2.59 ± 0.10	3.97 ± 0.26
20:4 ω 3	0.56 ± 0.03	0.49 ± 0.06
20:5 ω 3	1.29 ± 0.06	15.63 ± 0.78
21:5 ω 3	0.80 ± 0.01	ND
22:5 ω 6	1.19 ± 0.05	0.12 ± 0.22
22:5 ω 3	3.37 ± 0.26	1.49 ± 0.21
22:6 ω 3	5.37 ± 0.31	30.65 ± 1.33

[a] ND, not detected.

(**Figure 6**). The resulting metHb can react with hydrogen peroxide or lipid hydroperoxides to form a ferryl cation protein radical capable of inititating lipid oxidation (*50*). Alternatively, hemin is more readily released from metHb as compared to oxyHb and deoxyHb (*11*). Released hemin is capable of stimulating lipid oxidation by decomposition of preformed lipid hydroperoxides (*51*). The iron atom in deoxyHb is in a high-spin state, which makes deoxyHb more sensitive to oxidation as compared to the iron atom in oxyHb, which is in a low-spin state (*52*).

It must be noted that at pH 7.4, trout Hb autoxidized faster (**Figure 6**) and was a better promoter of lipid oxidation than tilapia Hb (**Figure 7**) in spite of the fact that Hb oxygenation

was equivalent in each heme protein at the elevated pH (**Table 1**). This indicates a mechanism other than deoxyHb-mediated metHb formation and subsequent lipid oxidation at pH 7.4. Zebrafish and *N. coriicep* Mb had nearly identical oxygen binding kinetics but very different autoxidation rates at equivalent pH (*53*). This also supports a mechanism of Hb autoxidation independent of oxygen affinity. One possible mechanism is increased entry of water molecules into the heme crevice of trout Hb as compared to tilapia Hb. This is in agreement with our CD spectral data, which suggest that trout Hb has a more open heme pocket. Coordinated water in the heme crevice is a reactant in Hb autoxidation (reaction 1). Using various Mb mutants, it has been shown that decreasing the size of amino acid residues near the heme crevice improves access of ligands (e.g., H_2O) to the heme crevice and accelerates autoxidation (*49*, *54*). Larger residue substitutions have the opposite effects. Tilapia Hb has not been sequenced, but variation in amino acid composition is common among different Hbs and Mbs. Heme pocket flexibility has also been shown to vary among different fish Mbs, which is believed to provide varying sized channels through which ligands can enter the heme crevice; those Mbs with increased flexibility near the crevice had more rapid autoxidation rates (*53*). The weaker heme−globin interactions in trout Hb as compared to tilapia Hb, as suggested by the CD spectra, and reduced overall secondary and tertiary structure and greater susceptibility to chemical and thermal denaturation are strong indications that trout Hb has a looser more flexible structure that tilapia Hb. It has also been shown that increased access of water to the heme crevice accelerates loss of hemin from metMb due to hydration of the proximal histidine−globin covalent bond (*55*).

Lipid oxidation and metHb formation occurred more rapidly at pH 6.3 as compared to pH 7.4 whether trout Hb or tilapia Hb was examined (**Figure 6** and **7**). One explanation is that Hb subunits form more readily at reduced pH (*56*). Subunits are more prone to metHb formation and release their hemin moiety more readily than tetramers (*57*, *58*). Our conformational studies demonstrated that subunit interactions were modified as the pH was reduced, which could suggest that part of the subunits dissociated. Decreasing the pH itself also increases hemin release. This is due to protonation of the proximal histidine, which weakens the heme−globin linkage (*40*), which was clearly shown by the pH 6.3 CD spectra. In human Hb, the hydrogen-bonding distance between liganded O_2 and the distal histidine are greater in β-chains as compared to α-chains. The greater distance in β-chains is believed to prevent a proton transfer mechanism by which metHb formation occurs (*59*). This can explain why α-chains autoxidize more rapidly than β-chains. A similar phenomenon in Hb autoxidation is possible when the pH is altered or when different Hbs are compared.

It is typical that increasing fatty acid unsaturation will increase rates of lipid oxidation because the more highly unsaturated lipids, with their higher number of 1,4-pentadiene units, are more prone to lipid oxidation (*60*). Interestingly, our results showed that Hb oxidized washed tilapia lipids more effectively than washed cod lipids in spite of the fact that washed cod lipids were around 2.8 times more unsaturated (**Table 3**). The order of oxidative stability was found to increase with an increasing degree of unsaturation when different fatty acids were dispersed in 1% Tween 20 and exposed to iron salts and ascorbate (*61*). This indicates that under certain conditions highly unsaturated fatty acids are more resistant to lipid oxidation than more saturated ones.

It should also be noted that the free fatty acid content was around 2.7 times greater in washed cod as compared to washed tilapia on a lipid basis (**Table 2**). Lipolysis effects in fish muscle indicated that phospholipid hydrolysis was inhibitory to lipid oxidation (*62*). Cabbage phospholipase D inhibited lipid oxidation in beef homogentates and egg yolk liposomes (*63*). Phospholipids were the main class of lipids present in washed cod (**Table 2**). A possible explanation for decreased lipid oxidation in washed cod includes a physical rearrangement of the lipids due to hydrolysis of phospholipids so that promoters of lipid oxidation have poorer access to the substrate. A complementary explanation involves the role of lysophospholipids. After one fatty acid is cleaved from a phospholipid, a lysophospholipid is formed. Lysophospholipids interact with Hb to form hemichromes (*64*). Hemichromes are considered less reactive as compared to other forms of Hb since the sixth ligand site of the heme is covalently bonded to an imidazole group (*65*). At the same time, it should be considered that on a muscle weight basis, washed cod and washed tilapia contained 0.058 and 0.097 g free fatty acids per 100 g muscle, respectively. The high percentage of triacylglycerols in washed tilapia (**Table 2**) suggests that some of the free fatty acids present were due to hydrolysis of the triacylglycerols. Previously, it was suggested that hydrolysis of triacylglycerols played a major role in the sensory deterioration of salmon during frozen storage (*66*). Hydrolysis of triacylglycerols was described to promote lipid oxidation in fish muscle (*62*).

The phospholipid content is likely equivalent in washed cod and washed tilapia because the membrane fraction should not be removed by washing. Apparently, the washing procedure did not effectively remove triacylglycerols from tilapia muscle (**Table 2**). The fact that the fat content was higher in washed tilapia as compared to washed cod is another factor that may have caused lipid oxidation to occur more rapidly in washed tilapia (i.e., there is more lipid substrate). However, it was previously shown that the rate and extent of lipid oxidation mediated by Hb was equivalent when washed cod substrate (around 0.7% phospholipid) was compared to the same washed cod that also had up to 15% menhaden oil triacylglycerols added (*67*). Triacylglycerols are more saturated and provide less surface area as compared to phospholipids in the membrane fraction.

The degree of membrane disruption in washed cod and washed tilapia was not assessed as part of these studies. However, electron microscopy images of different washed cod preparations indicated that washed cod with a disrupted myocomata was resistant to Hb-mediated lipid oxidation, while an intact myocomata oxidized readily (*68*). Less membrane disruption in washed tilapia as compared to washed cod may explain why Hb-mediated lipid oxidation was greater in the washed tilapia.

Although trout Hb was a better promoter of lipid oxidation than tilapia Hb in an unheated system, upon cooking, these two heme proteins were equivalent in their ability to promote lipid oxidation (**Figure 10**). Cooking temperatures cause rapid conversion of the heme protein from the ferrous to the met state. As noted above, hemin affinity is much higher in reduced Hb as compared to oxidized Hb. Furthermore, the globin unfolds and is denatured upon heating, which should weaken hemin affinity. Unfolding of Mb weakens the heme−globin linkage (*11*). Previously, it was shown that hemin affinity could be varied 975-fold by mutating sperm whale Mb at site 68 (V68T) and site 97 (H97A) (*40*). Furthermore, the ability of V68T, wild-type Mb, and H97A to oxidize washed cod lipids (unheated)

was consistent with their hemin affinities; high hemin affinity mutants were weak promoters of lipid oxidation, while low hemin affinity mutants were potent promoters (*12*). Thus, single amino acid substitutions can have profound effects on functional properties and lipid oxidation capacity in the raw state. However, any differences in amino acid composition between trout Hb and tilapia Hb that can have an effect on their relative abilities to promote lipid oxidation in the raw state may be lost when the different globins denature due to heating. Indeed, V68T, wild-type Mb, and H97A were equivalent in their ability to oxidize lipids in heated washed cod (*69*). Even though the thermal denaturation studies suggested that tilapia Hb was more stable than trout Hb, they were done at 50 °C, while the washed system was cooked at 80 °C. At 80 °C, denaturation and oxidation are expected to be much more rapid, so at that temperature any difference between the two proteins may be small, and subsequently, they may denature to the same extent within the time taken to cook the samples.

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