JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Lipid Oxidation in Trout Muscle Is Strongly Inhibited by a Protein That Specifically Binds Hemin Released from Hemoglobin

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ABSTRACT: The recombinant streptococcal protein apoShp can be used as a probe for hemoglobin (Hb) reactivity in fish muscle due to its specific affinity for hemin that is released from Hb at post-mortem pH values. Hemin affinity measurements indicated that apoShp binds hemin released from Hb but not myoglobin (Mb). Hemin affinity of holoShp was higher at pH 5.7 compared to pH 8.0. This may be attributed to enhanced electrostatic interaction of His58 with the heme-7-propionate at lower pH. ApoShp readily acquired hemin that was released from trout IV metHb in the presence of washed cod muscle during 2 °C storage at pH 6.3. This was based on increases in redness in the washed cod matrix, which occurs when apoShp binds hemin that is released from metHb. ApoShp prevented Hb-mediated lipid oxidation in washed cod muscle during 2 °C storage. The prevention of Hb-mediated lipid oxidation by apoShp was likely due to bis-methionyl coordination of hemin that dissociated from metHb. This hexacoordination of hemin appears to prevent peroxide-mediated redox reactions, and there is no component in the matrix capable of dissociating hemin from Shp. ApoShp was also added to minced muscle from rainbow trout (*Oncorhynchus mykiss*) to examine the degree to which Hb contributes to lipid oxidation in trout muscle. Addition of apoShp inhibited approximately 90% of the lipid oxidation that occurred in minced trout muscle during 9 days of 2 °C storage on the basis of lipid peroxide, hexanal, and thiobarituric acid reactive substances (TBARS) values. These results strongly suggest that Hb is the primary promoter of lipid oxidation in trout muscle.

KEYWORDS: *rancidity, heme proteins, mechanism, lipid oxidation, protoporphyrin IX*

INTRODUCTION

Lipid oxidation negatively affects the quality of muscle foods including defects such as product discoloration, undesirable flavors, and off-odors.[1] Hemoglobin (Hb) has been shown to promote lipid oxidation in washed muscle much more effectively compared to myoglobin (Mb) at pH 6.0, with lipid oxidation products reaching maximal values at day 1 of storage due to Hb, whereas lipid oxidation products due to Mb reached only about half that of Hb during 9 days of storage.[2]

Hemoglobin is a tetrameric protein found within erythrocytes of the blood, whereas myoglobin is a monomeric protein located inside myocytes. Each globin contains a prosthetic group that is termed ferroprotoporphyrin IX (or heme) when the central iron atom is in the +2 oxidation state or ferriprotoporphyrin IX (or hemin) when in the +3 oxidation state. Dissociation of hemin from carp metHb occurred readily at pH 6.0, whereas no hemin loss from carp metMb was observed at pH 6.0.[2] This suggested that the exceptional ability of Hb to promote lipid oxidation in washed muscle was due to release of hemin from the globin and incorporation into the lipid phase. Released hemin readily decomposes preformed lipid hydroperoxides (LOOH) into free radicals, which stimulate the oxidation of lipids.[3]

Previous work showed that the recombinant protein apoShp^{229} acquired hemin when mixed with metHb but not metMb from bighead carp at pH 6.0 during refrigerated storage.[4] The specific ability of apoShp^{229} to bind hemin that is released from metHb presents an opportunity to distinguish effects of Hb from Mb in muscle tissue that often contains varying concentrations of both heme proteins. ApoShp^{229} is a fragment of the native apoShp protein found in *Streptococcus pyogenes*.[5] ApoShp^{229} is composed of amino acids 30−258 of the native apoShp protein. The secretion signal sequence (amino acids 1−29) and the transmembrane domain and charged tail (259−291) are excluded. Shp^{180} is the hemin-binding domain of Shp, which includes amino acids 30−180.[6]

For apoShp to be used as a probe for Hb reactivity in muscle tissue, the hemin acquired from metHb must be nonreactive. The terms Shp and apoShp are used to describe the protein with and without bound protoporphyrin IX, respectively. There are two characteristics of Shp that may cause bound hemin to be inert. First, Shp exhibits bis-methionyl coordination of bound hemin.[6] This hexacoordination of hemin should prevent redox reactions with peroxides. Pentacoordination allows access of peroxide to the iron atom of the protoporphyrin IX, whereas hexacoordination blocks access.[7] Second, the hemin acquired by apoShp must not subsequently dissociate during storage in the muscle. This seems likely because a specific "plug-in" mechanism was necessary for the transfer of hemin from Shp to apoHtsA in the hemin acquisition machinery of *S. pyogenes*.[8] Studies utilizing apoShp as a probe for Hb reactivity in muscle

Received: February 6, 2013
Revised: April 5, 2013
Accepted: April 9, 2013
Published: April 9, 2013

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can take advantage of the absence of apoHtsA to ensure that hemin acquired from metHb remains inert and hexacoordinated in Shp during storage. With these considerations in mind, we wished to examine the ability of apoShp to affect lipid oxidation in finely comminuted muscle tissue.

■ MATERIALS AND METHODS

Preparation of Trout IV Hb. Blood was removed from the caudal vein of rainbow trout (*Onchorhyncus mykiss*) via syringe after exposure of trout to aminobenzoic acid ethyl ester anesthetic (0.5g/L water). Typically, 4 mL of blood was drawn into the syringe barrel preloaded with 1 mL of 0.9% NaCl containing 150 units of heparin. Four volumes of ice-cold 0.9% NaCl in 1 mM Tris, pH 8.0, was added to the anticoagulated blood and centrifuged (1000*g* for 10 min at 4 °C). After removal of the plasma, the erythrocytes were washed by suspension twice in 10 volumes of the above buffer. Cells were lysed in 3 volumes of 1 mM Tris, pH 8.0, for 1 h. NaCl was then added to bring the Hb and cell debris solution to 2% (w/v) NaCl to aid in stromal removal before ultracentrifugation (30000*g* for 1 h at 4 °C). The supernatant was then buffer exchanged into 50 mM Tris (pH 8.0) using a desalting column (GE Healthcare). Protein was then applied to a DEAE column equilibrated with the same buffer. Trout Hb I, II, and III[9] were passed through the column, and trout IV Hb was then eluted with 50 mM Tris−500 mM NaCl at pH 8.0.[10] Trout IV Hb was further purified through HiLoad 26/60 Superdex 200 pg (GE Healthcare) running with 10 mM Tris−25 mM NaCl (pH 8.0). Finally, the purified Hb was frozen in liquid nitrogen and stored at −80 °C. Electrospray ionization−mass spectrometry (ESI-MS) analysis indicated that the α and β chains of trout IV Hb were the only detectable polypeptides in the preparation (data not shown).

Preparation of MetHb. Four moles of potassium ferricyanide was added per mole of Hb (on a heme basis) and mixed. After incubation on ice for 1−2 h, metHb was passed through a Hitrap desalting column (5 × 5 mL) equilibrated with 10 mM Tris (pH 8.0). An OD_{280}/OD_{262} ratio of 1.1−1.2 was used to ensure sufficient removal of ferricyanide. The concentration of metHb (on a heme basis) was determined using a millimolar extinction coefficient of 153 mM^{-1} cm^{-1} at 405 nm.[11]

Preparation and Quantification of ApoShp^{229} and Shp^{180}. ApoShp^{229} was prepared from inclusion bodies as described previously.[12] The concentration of apoShp^{229} was determined using the extinction coefficient of 28.0 mM^{-1} cm^{-1} at 280 nm. Shp^{180} was prepared as described elsewhere.[6] The concentration of Shp^{180} was determined using the extinction coefficient of 15.2 mM^{-1} cm^{-1} at 280 nm.

Preparation of Wild-Type Mb and ApoH64Y. Wild-type sperm whale Mb and the sperm whale Mb mutant H64Y were expressed and purified as described previously.[13] Hemin was removed from H64Y with methyl ethyl ketone as described previously[14] with the following modifications. A 1 mL aliquot of H64Y was thawed and transferred into a test tube on ice. An equal volume of 0.2 M maleic acid (pH 2.2) was added into the protein solution and kept on ice for 10 min. After hemin extraction by methyl ethyl ketone, apoH64Y was loaded on a Hitrap desalting column 5 × 5 mL equilibrated with 10 mM Tris (pH 8.0) to remove residual organic compounds. For hemin loss measurement, the apo H64Y Mb was directly buffer exchanged into 150 mM bis-tris (pH 5.7) or Tris (pH 8.0) with 450 mM sucrose.

Hemin Loss Measurements. Hemin loss rates were determined by mixing 40 μM apoH64Y with 10 μM experimental heme protein at 25 °C. H64Y (containing bound hemin) exhibits a peak absorbance at 600 nm that is used in hemin loss rate calculations due to the unique absorbance of this Mb mutant.[15] Changes in absorbance at 600 nm were measured over time, and increases in turbidity at 700 nm were subtracted from each subsequent reading at 600 nm due to gradual precipitation of the apoH64Y or experimental apoprotein. Igor Pro software (WaveMetrics Inc., Portland, OR, USA) was used to calculate rates of hemin loss with the following assumptions: (i) In the presence of excess apoH64Y, all of the free hemin released from tested heme protein is acquired by apoH64Y. (ii) Denaturation to apoglobin can be neglected. With these assumptions, hemin transfer between tested met heme protein and apoH64Y is measured by

$$\mathrm{PH} \underset{k_{\mathrm{H}}}{\overset{k_{-\mathrm{H}}}{\rightleftharpoons}} \mathrm{YH} + \mathrm{P} \quad (1)$$

PH is the test protein (holo form), YH is the H64Y Mb (holo form), and P is the test protein (apo form). k_{-H} is the hemin loss rate from the test holo-protein, and k_H is the hemin association rate back to the test apoprotein. If the hemin transfer reaction is relatively slow, the normalized decay curve with time will follow a single exponential function and an apparent hemin transfer rate of $k_{obs} = k_{-H} + 2k_H[YH]_{eq}$ (eq 2). The equilibrium constant for hemin loss reaction is $K_{-H} = [YH]_{eq}^2/([PH]_{total} - [YH]_{eq}) = k_{-H}/k_H$ (eq 3). Hemin loss from met perch hemoglobin at the same temperature was set as reference assay to determine $[PH]_{total}$ because of its extraordinary ability to release hemin compared with other heme proteins. From eqs 2 and 3, hemin loss rate k_{-H} and other kinetic parameters are derived.

Preparation of Washed Cod Muscle. Fresh cod (*Gadus morhua*) fillets (around 2.5 lb) were obtained from The Seafood Center (Madison, WI, USA). Fillets were trimmed to remove all bones and dark tissue, cut into small pieces, and ground using a KitchenAid, Inc. (St. Joseph, MI, USA), KSM 90WW household mixer equipped with a grinding apparatus (5 mm plate diameter). Ground muscle was washed by combining with cold 50 mM sodium phosphate (pH 6.3) buffer (1:3 ratio) and mixing for 2 min with a glass rod. After settling for 15 min, muscle was collected and dewatered using a fiberglass screen. The above washing operation was repeated twice. After a final wash, the muscle slurry was homogenized for 3.5 min with a Polytron Type PT 10/35 probe (Brinkmann Instruments, Westbury, NY, USA) until it was of a fibrous consistency. The pH of the muscle slurry was checked and determined to be between 6.30 and 6.40. Muscle was collected in 50 g pellets by centrifugation for 25 min at 15263*g* and was then stored at −80 °C in vacuum-sealed plastic bags. All washing, dewatering, and centrifugation steps were performed at 4 °C.

Addition of ApoShp^{229} and MetHb to Washed Cod Muscle. Washed cod mince was thawed overnight at 4 °C and was crumbled into pieces by mixing for 10 min with a plastic spatula on ice. To further break up the mince, the washed cod was ground for three 5 s bursts at the medium 15 setting in a Hamilton Beach Custom Grind Type CM04 coffee grinder (Hamilton Beach/Proctor Silex, Inc., Southern Pines, NC, USA). The pH of the muscle was then adjusted to 6.3 if necessary by addition of 1 N HCl followed by mixing. Water was added to each reaction vessel so that the final moisture content was 90%, and streptomycin sulfate was added to a final concentration of 200 ppm to prevent microbial growth during storage. ApoShp^{229} (200 and 400 μM) was then added and mixed for 3 min with a plastic spatula followed by addition of metHb (40 μM on a heme basis). Hb in the absence of added apoShp^{229} was also examined. Samples were stored for up to 9 days at 2 °C.

Addition of ApoShp^{229} to Minced Trout Muscle. Fresh rainbow trout (*O. mykiss*) was obtained from Rushing Waters (Palmyra, WI, USA) and transported to the laboratory on ice. The following day, fillets from whole muscle (deskinned) were removed, cut into pieces, and ground using a KitchenAid, Inc., household mixer equipped with a grinding apparatus (5 mm plate diameter). The mince was then further comminuted in a Cuisinart Professional 14 chopper. The comminuted muscle was transferred into an amber reaction vial. Trout whole muscle contains approximately 40 μmol Hb/kg tissue on a heme basis.[16] Therefore, 200 μmol/kg tissue apoShp^{229} was added to the mince and mixed well using a plastic spatula. Milli-Q water was added so that the final moisture was 90%. Samples contained 200 ppm streptomycin sulfate to prevent microbial growth. Bovine serum albumin was also examined in place of apoShp^{229} (equivalent on a protein weight basis) to examine a protein that does not form bis-methionyl coordination with hemin that is released from metHb.

Color Measurement. Redness (expressed as *a* values) was measured with a Minolta CR-200 colorimeter (Minolta Camera Co., Osaka, Japan). A white calibration plate supplied with the unit was used to calibrate the instrument.[17]

Lipid Peroxides. Around 0.3 g of tissue was homogenized in 5 mL of chloroform/methanol (1:1) for 30 s using a Polytron Type PT 10/35 (Brinkmann Instruments). Subsequently, the Polytron 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 ammonium thiocyanate (4.38 M) and 25 μL of iron(II) chloride (18 mM) were added, and samples were incubated for 20 min at room temperature before the absorbance at 500 nm was determined.[18] A standard curve was prepared using cumene hydroperoxide. The chloroform used contained ethanol as a preservative to eliminate high blank readings.

Thiobarbituric Acid Reactive Substances (TBARS). TBARS were determined according to the modified method of Buege and Aust.[19] On the day of analysis, a solution of 50% trichloroacetic acid (TCA) with 1.3% thiobarbituric acid (TBA) was prepared by mixing and heating to 65 °C for 1 h; 80−120 mg of sample was added to 1.2 mL of the reagent. After heating at 65 °C for 60 min, the samples were cooled at 4 °C for 60 min. Samples were then centrifuged at 16000*g* for 5 min. Absorbances of supernatants were measured at 532 nm minus 650 nm. A standard curve was constructed using tetraethoxypropane, and concentrations of TBARS in samples were expressed as micromoles of TBARS per kilogram of washed muscle.

Hexanal Determination. Homogenized whole muscle (1 g) and internal standard (1 μL, 200 ppm chlorobenzene in methanol, Supelco, Bellefonte, PA, USA) were transferred into a 10 mL glass vial. A PTFE/silicone septa (MicroLiter Analytical Supplies Inc., Suwanee, GA, USA) was placed on the top of the vial, which was sealed with a 20 mm metal crimp. SPME fiber (65 μm thickness) with a stationary phase coating of polydimethylsiloxane−divinylbenzene, PDMS-DVB (Supelco), was used. PDMS-DVB was superior to Carboxen−PDMS and Carbowax−DVB in assessing hexanal in cooked turkey muscle.[20] The fiber was conditioned at 260 °C for 30 min in a gas chromatograph (GC, HP 6890, Hewlett-Packard, Palo Alto, CA, USA) injection port equipped with a capillary column (DB-5, 30 m length × 0.25 mm i.d. × 0.1 μL film thickness) and flame ionization detector (FID). Sealed vials containing homogenized muscle were preheated for equilibration at 40 °C for 5 min. The SPME fiber was then inserted in the headspace of the vial for 10 min at 40 °C to adsorb hexanal. After injection of the SPME fiber into the GC-FID injection port, hexanal was released from the fiber at 250 °C for 5 min. Helium as a carrier gas and splitless mode were used. The flow rate of carrier gas was 1 mL/min. Inlet and detector temperatures were 250 and 270 °C, respectively. The oven temperature was programmed at 40 °C for 5 min with a 10 °C/min ramp rate until 90 °C. Hexanal was identified by comparison of retention time of hexanal standard (Sigma-Aldrich, Steinheim, Germany). The quantity of hexanal in the sample was calculated using the area of the internal standard and comparisons to a standard curve constructed with the hexanal standard.

Statistical Evaluations. A MIXED procedure of the SAS system was used to analyze data from storage studies.[21] Means were separated using the *p*-diff test. For each treatment, two or three separate reactions were examined during storage. Because a subsample was removed from each reaction vessel at each time point, repeated measures were used. Significance was defined at $p < 0.05$.

■ RESULTS

The hemin dissociation rate (k_{-H}) from sperm whale metMb was 18-fold lower compared to that of Shp[180] at pH 5.7 (Table 1). Conversely, trout IV metHb had a 58-fold higher k_{-H} compared to Shp[180] at pH 5.7 and a 100-fold higher value at pH 6.3 (Table 1). These differences suggest that apoShp will readily bind hemin that is released from Hb and have no effect on the reactivity of Mb at post-mortem pH values. The k_{-H} from Shp[180] was 12-fold faster at pH 8.0 compared to that at pH 5.7 (Table 1). The hemin association rate (k_H) of Shp[180] was 6-fold faster at pH 8.0 compared to that at pH 5.7, which resulted in an equilibrium hemin dissociation constant (K_{-H}) that was 2-fold lower at pH 5.7. The relatively high k_H in sperm whale Mb resulted in a K_{-H} that was 83-fold lower in the Mb compared to that in Shp[180] at pH 5.7 (Table 1).

Table 1. Kinetic and Equilibrium Parameters for Hemin Loss from Different Heme Proteins at 25°C (pH 5.7 and 8.0)[a]

	pH	k_{-H} (h^{-1})	k_H (μM^{-1} h^{-1})	K_{-H}[b] (μM)
metMb	5.7	0.003 ± 0.001	0.08 ± 0.03	0.04 ± 0.002
Shp180	5.7	0.055 ± 0.001	0.016 ± 0.001	3.41 ± 0.27
Shp180	8.0	0.68 ± 0.004	0.10 ± 0.013	7.01 ± 0.88
metHb (trout IV)[c]	5.7	3.2 ± 0.05	nd[d]	nd
metHb (trout IV)[c]	6.3	5.5 ± 0.02	nd	nd

[a]Reactions contained metMb or Shp180 (10 μM), apoH64Y (40 μM), 150 mM BisTris (pH 5.7) or 150 mM Tris (pH 8.0), and sucrose (400 μM). The blank reaction contained apoH64Y and sucrose in Tris buffer at the concentrations shown. [b]K_{-H} is the equilibrium dissociation constant of hemin loss derived from k_{-H}/k_H. [c]From previous study at equivalent temperature and pH.[44] [d]nd, not determined.

The ability of apoShp[229] to bind hemin released from trout IV metHb was examined in washed cod muscle at pH 6.3 during 2 °C storage. ApoShp[229] was used rather than apoShp[180] due to the ease of preparing apoShp[229] from inclusion bodies after protein expression compared to removing hemin from purified Shp[180]. ApoShp[229] was added at a 5- and 10-fold ratio to metHb on a heme basis. Redness (*a* value) was used to monitor the transfer of hemin from metHb to apoShp in washed cod. A characteristic of the bis-methionyl ligation of hemin by Shp causes the protein complex to have a characteristic red color.[8] MetHb is a brown pigment so that increases in redness are indicative of hemin transfer from metHb to apoShp[229]. Redness values increased between 0 and 12 h in samples containing metHb and apoShp[229], which indicated that transfer of hemin from metHb to apoShp occurred (Figure 1). After 12 h, there was a gradual decline in redness in the presence of apoShp during 4 days of storage, although values did not decline below initial values.

In the absence of added apoShp[229], there was a rapid decrease in *a* value to negative values during the initial 2 days of storage in washed cod containing added metHb (Figure 1). The sharp loss of pigmentation was likely due to degradation of the protoporphyrin IX ring. In the absence of apoShp, dissociated hemin is free to react with preformed LOOH. Free radicals derived from hemin-mediated LOOH decomposition degrade the protoporphyrin ring so that chromophore characteristics are removed.[22]

The ability of apoShp[229] to inhibit Hb-mediated lipid oxidation in washed cod was examined during 2 °C storage at pH 6.3. ApoShp[229] was added at a 5- and 10-fold ratios to metHb on a heme basis. MetHb caused rapid TBARS formation in the absence of apoShp, with TBARS reaching maximum values at day 2 of storage (Figure 2). In the presence of apoShp, TBARS formation was completely prevented during the entire storage period at both the 5- and 10-fold ratios to metHb.

The ability of apoShp[229] to inhibit lipid oxidation in minced and chopped trout muscle was examined during 2 °C storage. ApoShp[229] was added at 200 μmol/kg tissue, which was

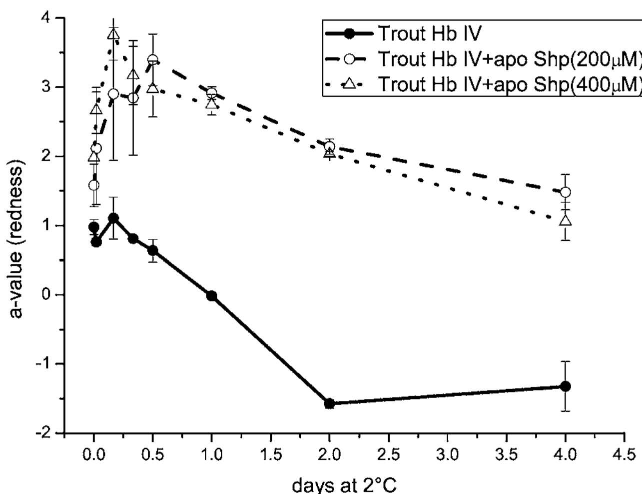


Figure 1. Redness values in the presence and absence of apoShp[229] in washed cod muscle that contained metHb during storage at 2 °C. The concentration of trout IV metHb was 10 μM (40 μM on a heme basis). pH was 6.3.

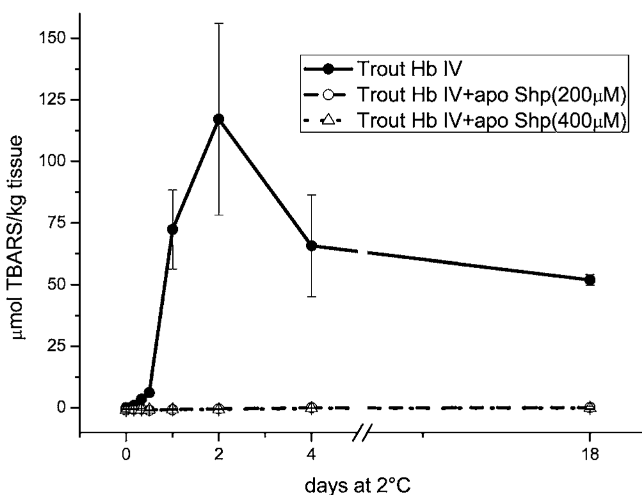


Figure 2. Ability of apoShp[229] to prevent hemoglobin-mediated lipid oxidation during storage at 2 °C based on TBARS values. The concentration of trout IV metHb was 10 μM (40 μM on a heme basis). pH was 6.3.

estimated to provide a 5-fold ratio of apoShp to metHb (on a heme basis) based on previous determination of Hb concentration in trout whole muscle.[16] Primary (lipid peroxides) and secondary (TBARS and hexanal) lipid oxidation products were measured. The trout muscle was thoroughly ground to maximize disruption of capillaries and erythrocytes in the muscle so that added apoShp would have access to endogenous Hb. In the absence of apoShp, TBARS values reached a plateau at day 5 of storage (Figure 3). Addition of apoShp (200 μmol/kg tissue) strongly suppressed TBARS formation in the ground trout muscle ($p < 0.05$). TBARS values at day 8 of storage were 4 μmol/kg in the presence of apoShp compared to 34 μmol/kg in its absence (Figure 3).

The ability of apoShp to affect hexanal formation was evaluated in ground trout muscle during 9 days of 2 °C storage. In this trial, the apoShp concentration was 153 μmol/kg tissue. TBARS values at day 3 were similar in control compared with apoShp-added samples. However, at days 6 and 9, apoShp strongly suppressed hexanal formation (Figure 4). Hexanal

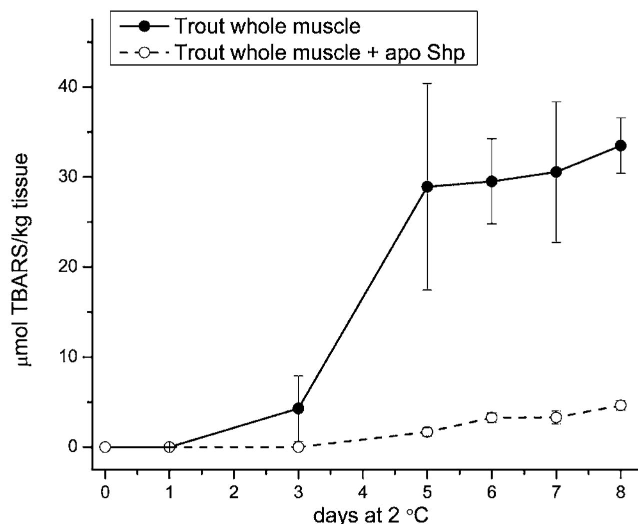
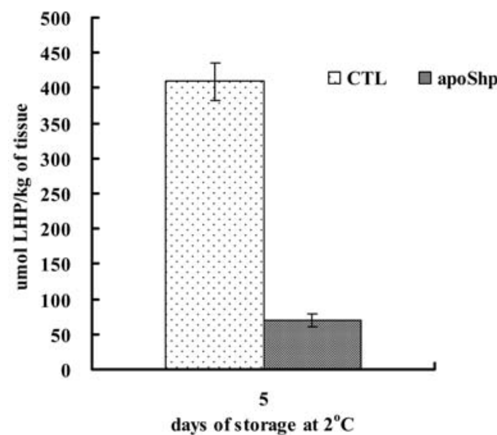


Figure 3. Ability of apoShp to inhibit lipid oxidation in minced and chopped trout whole muscle during storage at 2 °C based on thiobarbituric acid reactive substances (TBARS) values. ApoShp was added at 200 μmol/kg tissue.



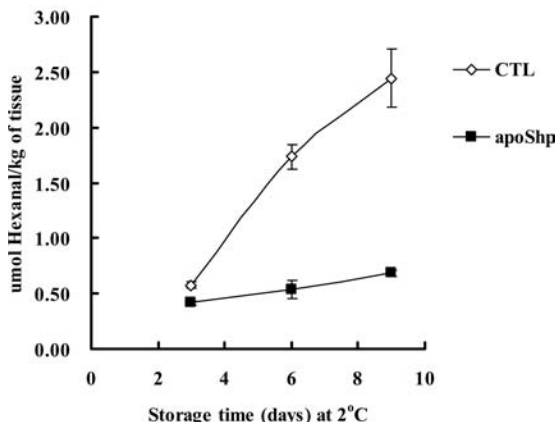


Figure 4. Ability of apoShp to inhibit lipid oxidation in minced and chopped trout whole muscle based on lipid peroxide values at day 5 and hexanal values during 9 days of storage at 2 °C. ApoShp was added at 153 μmol/kg tissue.

values at day 9 of storage were 0.6 μmol/kg in the presence of apoShp compared to 2.5 μmol/kg in its absence. ApoShp strongly inhibited lipid peroxide values in ground trout muscle at day 5 of 2 °C storage. Lipid peroxide values at day 5 of

dx.doi.org/10.1021/jf4006142 | J. Agric. Food Chem. 2013, 61, 4180−4187

storage were 65 μmol/kg in the presence of apoShp compared to 420 μmol/kg in its absence (Figure 4).

The ability of added bovine serum albumin (BSA) to affect the formation of TBARS in macerated trout muscle was also determined during 2 °C storage. Proteins have the potential to inhibit lipid oxidation nonspecifically via free radical scavenging and metal chelation properties.[23,24] Thus, BSA was examined as a reactant that might inhibit lipid oxidation nonspecifically. BSA was added at 75 μmol/kg tissue to provide an equivalent amount of protein on a weight basis compared to experiments in which apoShp[229] was added. Addition of BSA had no effect on lipid oxidation in minced and chopped trout muscle during 8 days of storage (Figure 5).

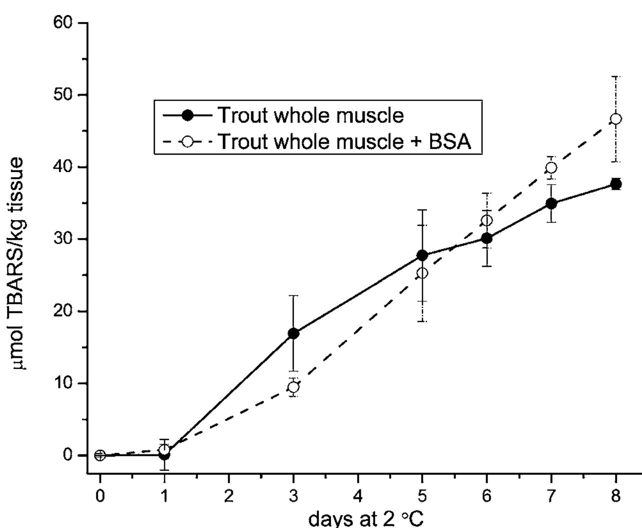


Figure 5. Thiobarbituric acid reactive substance (TBARS) values in minced and chopped trout whole muscle containing bovine serum albumin (BSA). BSA was added at 75 μmol/kg tissue to provide an equivalent amount of protein on a weight basis compared to apoShp addition in Figure 3.

■ DISCUSSION

The Ability of ApoShp To Strongly Inhibit Lipid Oxidation in Comminuted Trout Muscle Suggests That Endogenous Hb Is the Primary Reactant. There are an abundance of other potential reactants in muscle tissue that can facilitate lipid oxidation such as low molecular weight metal complexes (e.g., Fe-ADP), myoglobin, P450 reductase, myeloperoxidase, and lipoxygenase.[7,25] However, apoShp should have negligible effects on these reactants. The equilibrium hemin dissociation constant of sperm whale Mb was 85-fold lower than that of Shp at pH 5.7 (Table 1). Thus, apoShp should not acquire hemin from any trout Mb present in the muscle. The ability of apoShp to acquire hemin from bighead carp Hb but not carp Mb at pH 6.0 has been shown.[4] At the other extreme, the hemin dissociation rate from trout metHb was 58−100-fold faster compared to that from Shp at post-mortem pH values (Table 1). Thus, apoShp readily acquired hemin from trout metHb, which prevented Hb-mediated lipid oxidation in the washed cod muscle model system (Figure 2) and strongly inhibited lipid oxidation in comminuted trout muscle (Figures 3 and 4).

The Small Degree of Lipid Oxidation That Occurred in Trout Muscle Containing ApoShp Suggests There Is a Minor Contribution to Lipid Oxidation by Reactants Other than Endogenous Hb. Bighead carp Mb was shown to promote lipid oxidation in washed muscle to a small degree compared to bighead carp Hb during iced storage.[2] This may indicate that the small amount of lipid oxidation that occurred in comminuted trout muscle in the presence of apoShp was due to endogenous Mb. It should be noted that Hb was the only heme pigment determined in extracts from trout whole muscle.[16] The dilution and chromatography steps during quantification likely caused the small amount of Mb in the muscle to be undetectable. Endogenous lipoxygenases have the potential to make a contribution to the total lipid oxidation observed in trout muscle. Lipid oxidation due to Hb was more extensive than lipoxygenase in a model system of silver carp mince.[26] The low (<5 kDa) and high (<5 kDa) molecular weight fractions in the soluble phase of mackerel muscle weakly promoted lipid oxidation in liposomes compared to the combined fractions.[27] This indicated an interaction between high and low molecular weight components was required for maximal lipid oxidation in the model system. In any event, the strong inhibition of lipid oxidation in minced trout muscle (rather than a model system) by apoShp strongly suggests that endogenous Hb is the primary facilitator of lipid oxidation in the muscle.

There Are at Least Four Factors That Can Explain the Remarkable Ability of ApoShp To Prevent Hb-Mediated Lipid Oxidation in Comminuted Trout Muscle. First, bis-methionyl coordination of hemin in Shp results in a hexacoordinate ligation. The ability of H_2O_2 and LOOH to convert a heme protein to a peroxidase that can oxidize lipids relies on pentacoordination of prototoporphyrin IX.[28] Hemopexin (bis-histidyl coordination) is an example of a hexacoordinated hemin-transport protein that is considered to be nonreactive.[29] Hexacoordination does not alone guarantee a nonreactive heme protein. Cytochrome *c* exhibits hexacoordination (Met/His) yet catalyzed lipid oxidation in liposomes.[30] The postulated mechanisms of lipid oxidation by cytochrome *c* included the ability of excess H_2O_2 to degrade the protopophyrin ring and hemin dissociation in the presence of negatively charged liposomes. Neuroglobin contains bis-histidyl coordination in its crystal structure yet also converts to pentacoordination to bind nitrite.[31] It should be noted that Shp and bacterioferritin are the only known proteins that provide bis-methionyl coordination of hemin.[32] Our results indicate that bis-methionyl coordination renders the hemin obtained from metHb to be essentially nonreactive in post-mortem trout muscle.

Second, the hemin acquired by apoShp does not appear to dissociate during refrigerated storage because no component in the muscle is capable of displacing hemin from Shp. The transfer of hemin from Shp to apoHtsA in *S. pyogenes* is highly specific. A binary complex of Shp and apoHtsA has been described in which the two axial ligands of apoHtsA simultaneously displace hemin from the two axial ligands of Shp.[8] Adding apoShp to trout muscle in the absence of apoHtsA provides a framework to remove hemin from the highly reactive metHb and render that hemin nonreactive within Shp. Retention of hemin in Shp will prevent hemin-mediated decomposition of preexisting lipid hydroperoxides (LOOH) to free radicals that can propagate lipid oxidation.[33] The ability of hemin released from metHb to rapidly intercalate into phospholipids containing LOOH has been described.[34−37] ApoShp prevents the intercalation of hemin into phospholipids

dx.doi.org/10.1021/jf4006142 | J. Agric. Food Chem. 2013, 61, 4180−4187

by efficiently acquiring hemin from metHb in the minced and chopped trout muscle.

A third factor involves the electrostatic charge of Shp containing hemin. Shp is an anionic protein (p*I* 5.3), which indicates a net negative charge at post-mortem pH values. Cellular membranes are considered to be negatively charged due to the presence of negatively charged phospholipid head groups such as phosphatidylserine, phosphatidic acid, and phosphatidylinositols.[38,39] Thus, electrostatic repulsive forces may limit the ability of Shp that contains hemin to be drawn to negatively charged lipid domains in the muscle tissue.

A fourth factor involves the higher hemin affinity of Shp at post-mortem pH values compared to elevated pH (Table 1). The higher hemin affinity of Shp at lower pH values may be due to protonation of His58 as the pH is lowered. Protonation of His58 should increase the electrostatic interaction of His58 with the heme-7-propionate of Shp containing bound hemin (Figure 6). Elevated hemin affinity of Shp at post-mortem pH values will limit the ability of biomolecules or structures in trout muscle to extract hemin from Shp.

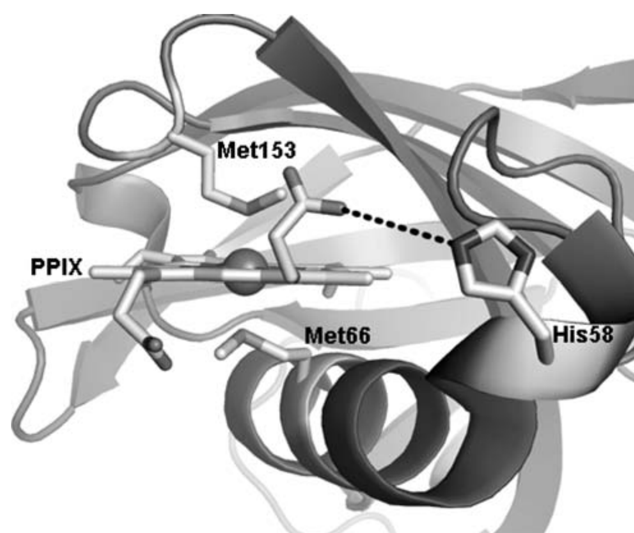


Figure 6. Streptococcal heme-associated protein (Shp) containing protoporphyrin IX (PPIX). His[58] of Shp can electrostatically bond with the heme-7-propionate group of PPIX. Enhanced protonation of His[58] at low pH values may explain the increased hemin affinity at pH 5.7 compared to pH 8.0. The bis-methionyl coordination of PPIX is shown. His[58], Met[66], Met[153], and PPIX are shown in stick representation. The iron atom of PPIX is shown as a sphere. PyMOL software was used to create the image shown using PDB 2Q7A.[6]

It Remains Unknown If There Are Protein−Protein Interactions between ApoShp and Trout IV MetHb prior to Hemin Exchange. The rates of hemin transfer from human metHb to apoShp at pH 8.0 were similar to those of simple hemin dissociation from the metHb, which suggested a protein−protein interaction did not occur.[40] It is interesting to note that Shp (pdb 2Q7A) and human metHb (pdb 1JY7) are both negatively charged at pH 8.0 (−22.5 and −23.4, respectively) yet have opposite charge at pH 6.0 (−16.9 and 43.8, respectively) based on PROPKA 3.1 analysis.[41] This suggests there may be electrostatic protein−protein interactions between Hbs and apoShp at post-mortem pH values that do not occur at elevated pH. Similar to human metHb, the electrostatic charge of trout IV metHb (pdb 3BOM) is also negative at pH 8.0 and positive at pH 6.0 on the basis of PROPKA analysis.

The Redox State of the Iron Protoporphyrin IX within Shp Is of Interest Because Affinity Is Usually Affected by the Oxidation State of the Central Iron Atom. The protoporphyrin IX binding protein Shr was found to bind hemin and reduce the bound hemin to heme even in the presence of air.[42] This may also be an attribute of Shp. Shp containing heme and hemin exhibit Soret peaks at 428 and 419 nm, respectively.[8] A Soret peak at 419 nm provides red hues, whereas a Soret peak at 428 nm provides relatively purple hues.[43] Thus, Shp containing hemin will exhibit more redness compared to Shp containing reduced heme. Redness decreased gradually from day 1 to day 4 in washed cod containing Shp that had acquired hemin from added metHb earlier in storage (Figure 1). The significance of Shp reducing acquired hemin to heme is that the ability of components or conditions in muscle to displace protoporphyrin IX from Shp may be particularly hindered in the event that the heme−Shp complex is stronger than the hemin−Shp complex.

The Physical Heterogeneity of Finely Comminuted Trout Muscle May Cause Concern That Added ApoShp Would Not Be Able To Effectively Acquire All of the Hemin Released from Endogenous Hb. It is important to recognize that metHb will form relatively slowly from oxyHb and deoxyHb in post-mortem trout muscle as endogenous reducing capacity is depleted during iced storage. This presents an opportunity for the added apoShp to readily acquire hemin from metHb due to the large molar excess of apoShp to *metHb* in the muscle at a single point in time. The ratio of apoShp to total Hb on a heme basis was ∼4:1. This large molar excess of apoShp to metHb combined with the 83−100-fold higher hemin dissociation rate from trout IV metHb compared to Shp at pH 5.7−6.3 (Table 1) helps to explain the remarkable ability of added apoShp to prevent Hb-mediated lipid oxidation in finely comminuted trout muscle.

A Remaining Question Involves the Ability of Dissociated Hemin To Decompose Preformed LOOH as the Primary Mechanism by Which Fish Hbs Promote Lipid Oxidation. This seems likely considering the 10−55-fold lower hemin affinity of fish Hbs compared to mammalian Hbs at post-mortem pH values.[44] The similar polarity of hemin and phospholipids combined with the rapid reaction rates of hemin with preformed LOOH provides a rationale for rapid onset of lipid oxidation upon hemin dissociation. Mb and Hb mutants with decreased hemin affinity were found to more effectively promote lipid oxidation compared to those with higher hemin affinity.[13,45,46] Further evidence for this pathway results from the ability of apoShp to bind hemin that is released from trout metHb, strongly inhibiting lipid oxidation during storage of the minced trout muscle. Future work should determine if specific interactions occur between metHbs and apoShp at post-mortem pH values or if, alternatively, only spontaneously dissociated hemin is acquired by apoShp. The ability of apoShp to inhibit lipid oxidation in beef, pork, and poultry should also be examined to differentiate between Mb- and Hb-mediated lipid oxidation that occurs during storage in the respective types of muscle foods.

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Funding

This project was supported by National Research Initiative Grant 2005-35503-16134 from the USDA Cooperative State Research, Education, and Extension Service Improving Food Quality and Value program and by the College of Agricultural and Life Sciences, University of Wisconsin—Madison, HATCH project 142PRJ28VY. Shp preparation was supported by Grant GM103500-09 from the National Institutes of Health.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

ADP, adenosine diphosphate; apoShp, form of Shp without bound heme; Hb, hemoglobin; holoShp, form of Shp with bound heme; H64Y, Mb mutant with tyrosine at site 64; HtsA, heme transporter of group A streptococcus; Mb, myoglobin; metHb, oxidized form of Hb; PPIX, protoporphyrin IX; LOOH, lipid hydroperoxide; Shp, streptococcal heme-associated protein; TBARS, thiobarbituric acid reactive substances

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