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Properties of Tilapia Carboxy- and Oxyhemoglobin at Postmortem pH

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Hemoglobin plays an important role in the color and oxidative stability of seafoods. A recent practice in the seafood industry is to stabilize muscle color by the application of gases containing carbon monoxide. The goal of this study was to examine and compare the properties of tilapia hemoglobin complexed to either O_2 (Oxy-Hb) or CO (CO-Hb) at pH 6.5, which reflects the tilapia muscle postmortem pH. CO-Hb was significantly ($p < 0.01$) more stable against autoxidation compared to Oxy-Hb when kept at 4 and −30 °C for 23 days. Almost no loss of CO was detected for both temperatures according to the UV−vis spectra of Hb. This stabilization was also believed to play a role in increased protein structure stabilization ($p < 0.001$) since less protein aggregation was seen for CO-Hb. The higher protein stabilization for Hb was linked to the heme group, which was maintained in its reduced state longer for CO-Hb vs Oxy-Hb and was likely less exposed to solvent. CO-Hb had significantly ($p < 0.01$) less peroxidase activity than Oxy-Hb and thus reactivity with H_2O_2. The pro-oxidative activity of CO-Hb was significantly ($p < 0.01$) reduced in a linoleic acid micelle system compared to that of Oxy-Hb, while smaller differences in activity were seen in a washed cod and tilapia muscle model system.

KEYWORDS: Hemoglobin; tilapia; CO (carbon monoxide); O_2 (oxygen); autoxidation; stability; peroxidase; pro-oxidative; lipid oxidation

INTRODUCTION

Heme proteins play an important role in the quality of aquatic foods. There are two key heme proteins in fish muscle, hemoglobin (Hb), which is located in the blood cells, and myoglobin (Mb), which is located within the muscle cell. Hemoglobin has been found in higher amounts than myoglobin in white muscle, while concentrations of both vary in dark muscle, depending on the species (*1*−*3*). Both proteins can be released and/or activated on processing and lead to significant quality problems in fish muscle. Most problems associated with heme proteins are due to their heme group (four in Hb and one in Mb), which contains an iron molecule that can be bound to different gas ligands in its reduced state. Autoxidation of the heme, i.e., conversion from the reduced Fe^{2+} state to the oxidized Fe^{3+} state, contributes greatly to undesirable color changes in aquatic foods. This is because the oxidized heme proteins have a brown appearance rather than the desirable fresh red color of reduced Oxy-Hb/Mb. This especially applies to the dark muscle of fish due to its higher levels of heme proteins than white muscle. Oxidized heme proteins are believed to be more pro-oxidative than reduced heme proteins under most circumstances (*4*), thus leading to rancidity problems in fish muscle which is rich in unstable polyunsaturated fatty acids. Recent evidence however points to a pro-oxidative activity of reduced heme proteins.

Being able to maintain heme proteins in their reduced state is hypothesized to have a positive impact on fish muscle quality. One way of maintaining heme proteins in their reduced state is to complex the heme with carbon monoxide (CO). CO binds to the heme with great affinity and will readily replace O_2 from the heme. The application of treating fish with gases containing CO is now widespread globally, with the purpose of stabilizing and in some cases enhancing the red color of seafood products, especially those directed to the frozen market. In this process, fish fillets and steaks are placed in chambers containing CO levels of from 18% to 100% for up to 48 h to allow for sufficient CO binding to the heme proteins. The CO binding changes the UV−vis spectra of the heme proteins, resulting in a cherry red color which is very stable on both frozen and refrigerated storage. Tilapia is now one of the most common species treated with CO. Tilapia is treated by either exposing its fillets to CO for a time period of about 30 min immediately after harvest and filleting or incorporating CO into fish tanks as a euthanasia method. Very limited scientific information is available on how CO influences product quality via the heme proteins. Kristinsson and co-workers (*5*, *6*) have shown that CO has a significant stabilizing effect on the hemoglobin protein structure under a variety of different conditions. It has also been reported that CO treatment leads to less lipid oxidation in fish muscle (*7*, *8*).

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Red color stability of the muscle of several fish species was also significantly stabilized with CO application (*7*−*10*). Since a majority of the CO in fish muscle is bound by the heme proteins, there is a great need to understand how this gas influences the functionality and stability of these proteins. The objective of this study was to investigate how the stability and activity of tilapia Hb were affected by CO treatment in comparison to those of Oxy-Hb.

MATERIALS AND METHODS

Oxy- and CO-Hemoglobin Preparation. Tilapia (*Oreochromis niloticus*) was obtained locally from a tilapia aquaculture farm (Evans Farms, Pierson, FL). Tilapia was placed on ice (pectoral side facing up) for 1 min and blood drawn via the caudal vein. Oxyhemoglobin was extracted from the red blood cells and purified according to the procedure of Fyhn et at (*11*). The purity of the Oxy-Hb preparation was verified by SDS−PAGE (*12*) and densitometry, which demonstrated that the samples were >99% hemoglobin. The samples were frozen in 0.5 mL aliquots in cryovials at −80 °C and thawed under 20 °C running water (<30 s) prior to all experiments. Hemoglobin was kept on ice during all experiments. Hemoglobin levels were quantified spectrophotometrically as described previously (*13*) with bovine hemoglobin as the standard. CO-Hb was prepared from Oxy-Hb by taking a 100 μL aliquot of Oxy-Hb, placing it in a 1 mL glass vial (on ice), and passing a stream of 100% CO over the solution for 2 min. The presence of CO-Hb was verified from the UV−vis spectra of the diluted CO-Hb solutions.

Autoxidation of Hemoglobin. Hemoglobin (10 μL) was added to amber cryovials (Fisher Scientific, Fair Lawn, NJ) containing 3 mL of 50 mM sodium phosphate buffer (4 °C) at pH 6.5. One set of the hemoglobin solutions were then placed at 4 °C for 23 days and regularly scanned between 350 and 700 nm in an Agilent 8453 diode array spectrophotometer (Agilent Technologies, Palo Alto, CA). Another set of the hemoglobin solutions were placed at −30 °C and sampled regularly for up to 23 days. The frozen solutions were thawed under running water (20 °C) for about 30 s before they were scanned in the spectrophotometer. Autoxidation was calculated from the UV−vis spectra of the hemoglobin solutions according to the equations by Krzywicki (*14*) and the percent autoxidation reported.

Protein Aggregation. Hemoglobin solutions were prepared and stored as described in Autoxidation of Hemoglobin. Protein aggregation was assessed by the absorbance of the protein at 350 nm (*15*) compared to a freshly prepared solution of unaggregated hemoglobin according to the equation

$$\text{relative protein aggregation (\%)} = [(A_{start} - A_t)]/[(A_{start} - A_{end})] \times 100$$

where A_{start} = absorbance at 350 nm of fresh hemoglobin solution at the start of the experiment, A_t = absorbance at 350 nm of hemoglobin at a given time point during storage, and A_{end} = absorbance at 350 nm of hemoglobin that had the highest level of aggregation of all Hb solutions (solution kept for 23 days at −30 °C). This equation does not give the absolute level of protein aggregation, but rather demonstrates a relative change in protein aggregation.

Peroxidase Activity of Hemoglobin. The peroxidase activity of tilapia hemoglobin was conducted as described by Fedeli et al. (*16*) with modifications. Hemoglobin (5 μL) was added to 900 μL of a buffer (pH 6.5) containing 50 mM sodium phosphate and 10 mM guaiacol. The assay was initiated by adding 100 μL of H_2O_2 (176 mM) with immediate mixing (<1 s). The change in absorbance at 470 nm was then immediately followed in an Agilent diode array spectrophotometer (Agilent Technologies, Palo Alto, CA).

Pro-oxidative Activity of Hemoglobin. Different systems were used to assess the pro-oxidative activity of CO- and Oxy-Hb. The first system consisted of linoleic acid (20 mg) and Tween 20 (20 mg) which were mixed by stirring with a plastic rod and diluted in distilled deionized water to give 2 mL. This solution was emulsified by forcing the solution through a pipet (1 mL) tip five times. The linoleic acid/Tween 20 solution (150 μL) was mixed with 850 μL of sample buffer (25 mM sodium phosphate at pH 6.5) and 10 μL of hemoglobin added to give a hemoglobin concentration of ~2 μM. Controls were run without hemoglobin. All assays were performed at 4 °C. Development of lipid oxidation was followed by measuring the rate of conjugated diene formation during a 50 min reaction period by recording absorbance changes at 234 nm using an Agilent 8453 diode array spectrophotometer (Agilent Technologies, Palo Alto, CA).

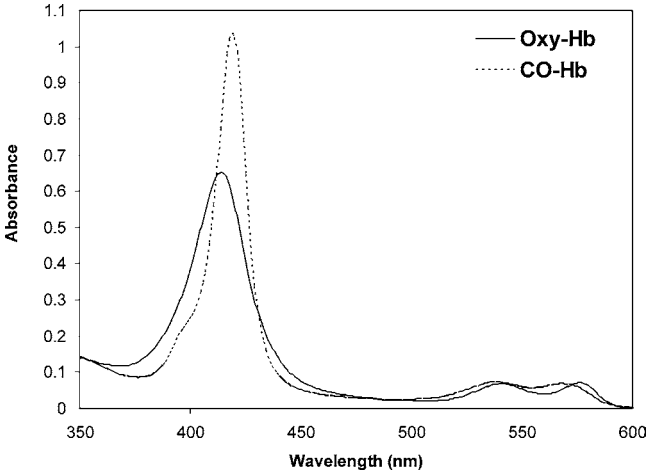

Figure 1. UV–vis spectra of tilapia Oxy- and CO-Hb at pH 6.5 in sodium phosphate buffer.

The second system used to assess oxidation was a washed fish muscle system prepared according to Richards and Hultin (*17*). Two fish muscle systems were used, cod and tilapia. Whole gutted cod was obtained on ice overnight from Steve Connolly's Seafood (Boston, MA) and immediately processed at the laboratory. Tilapia was obtained from a local aquaculture farm (Evans Farms, Pierson, FL) and processed on arrival at the laboratory. Both fish where ~48 h postmortem and had been kept on ice at all times. The fish were filleted and skinned. Special care was then made to remove all dark muscle and blood spots. The fillets were then cut into ~1 in. pieces which were ground in an Oster heavy-duty food grinder (Sunbeam Products Inc., Boca Raton, FL). The ground muscle was then suspended in 3 volumes of water and the suspension stirred every 5 min for 1 min for a total of 15 min. The slurry was then passed through clean cheesecloth and the recovered washed muscle subjected to the same procedure twice, with the last wash being in a 50 mM sodium phosphate solution (pH 6.5). The recovered washed muscle was then adjusted to pH 6.5 by dropwise additions of HCl and NaOH (1 M) and mixing with a plastic spatula. The pH of the systems was checked and verified by dissolving a 1 g sample in 10 mL of distilled deionized H_2O. After pH adjustment the moisture content of the washed muscle was determined with a Cenco moisture balance (CSC Scientific, Farifax, VA) which registered an average value of ~86%. Hemoglobin was added to the washed muscle matrix and the resulting solution mixed with a plastic spatula to give a concentration of ~6 μmol/kg of substrate. This system was spread on Petri dishes (~5 mm thickness) and kept at 4 °C with the lid on. Samples were periodically taken and measured for thiobarbituric acid reactive substances (TBARSs) according to Lemon (*18*) as modified by Richards and Hultin (*17*) and lipid hydroperoxides as described by Richards and Hultin (*17*).

RESULTS AND DISCUSSION

Autoxidation. The UV−vis spectrum was modified on CO binding, with the heme group shifting from 414 nm for Oxy-Hb to 419 nm for CO-Hb and also resulting in a more narrow and higher heme peak (**Figure 1**). This shifting is due to a different binding of the CO to the heme compared to that of O_2 and can be used as an indicator of the presence of CO in muscle foods (*7*). The two peaks between 520 and 600 nm indicative of bound gas ligands were blue shifted on CO binding (**Figure**

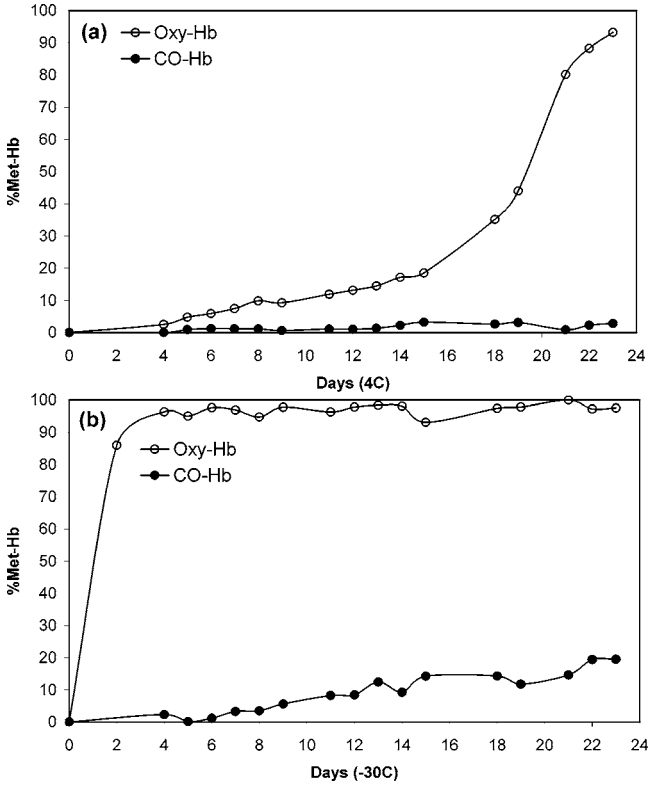

Figure 2. Autoxidation of tilapia Oxy- and CO-Hb reported as percent met-Hb formed at pH 6.5. Samples were kept for 23 days at (**a**) 4 °C or (**b**) −30 °C, and autoxidation was calculated on the basis of changes in absorbance.

1). It is clear from **Figure 2** that hemoglobin became significantly ($p < 0.01$) stabilized against autoxidation after CO was bound to the heme group, which is in agreement with previous findings (*6*, *19*). Oxy-Hb started to oxidize after only a few days at 4 °C and was close to fully oxidized after 23 days of storage, while CO-Hb remained largely unoxidized throughout the storage period (**Figure 2a**). This remarkable oxidative stability of CO-Hb was also seen during frozen storage, where Oxy-Hb oxidized almost completely within 4 days while almost none of the hemoglobin in the CO-treated samples was oxidized (**Figure 2b**). Even after 23 days of frozen storage only about 19.5% of the CO-Hb preparations were oxidized. It is possible that the thawing process along with the frozen storage contributed in part to the autoxidation of Oxy-Hb compared to CO-Hb. When the shift in heme group absorbance was monitored (**Figure 3**), it could be seen that the peak wavelength exhibited a blue shift for Oxy-Hb which stabilized at 405 nm. This signifies a loss of oxygen and autoxidation which is further supported by a disappearance of the characteristic oxygen binding peaks (between 520 and 600 nm) of Oxy-Hb (**Figure 4a**). The heme peak was also broadened and reduced in intensity, indicating a more open and presumably unstable heme pocket (*20*, *21*) (**Figure 4a**). The heme peak did not shift for CO-Hb during 4 or −30 °C storage, indicating that CO was still bound to the heme and the configuration of the heme pocket thus less affected. However, since autoxidation was found to some extent for CO-Hb samples subjected to frozen storage, it must be concluded that some of the CO was lost from Hb at −30 °C. This might be explained by the small drop in the heme peak (**Figure 4b**), thus indicating that part of the hemoglobin most likely lost its CO. A roughly 1.6-fold increase in the heme peak is observed on CO binding (compare the peaks in **Figure 1**),

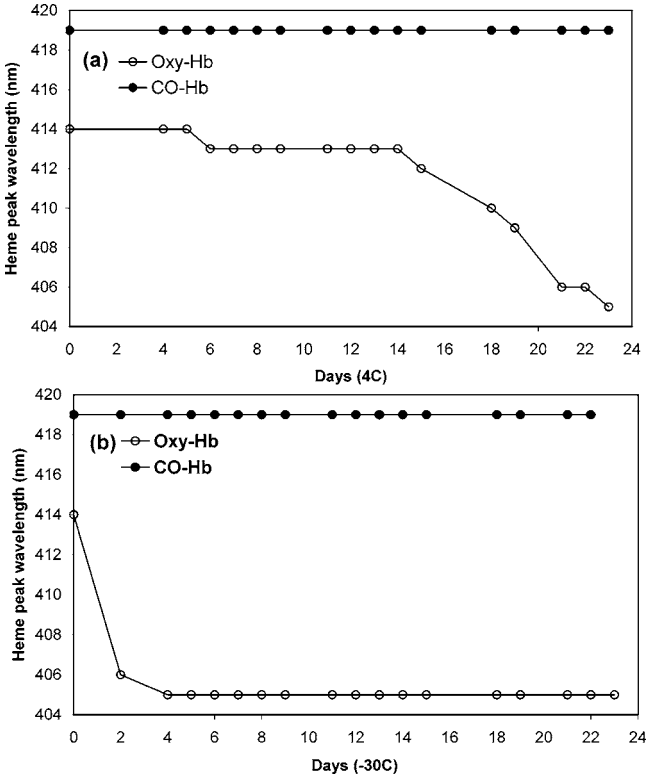

Figure 3. Change in the heme peak wavelength of tilapia Oxy- and CO-Hb over 23 days at (**a**) 4 °C or (**b**) −30 °C. Hemoglobin solutions were at pH 6.5.

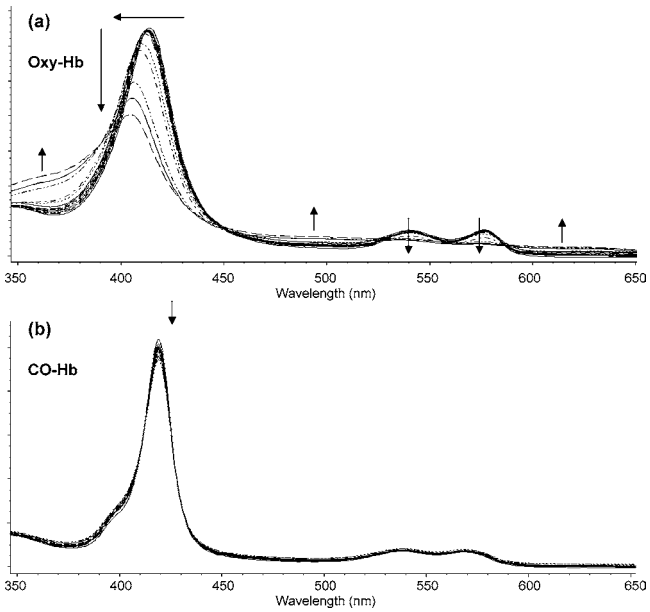

Figure 4. Changes in the UV–vis spectra of tilapia (**a**) Oxy-Hb and (**b**) CO-Hb over 23 days at 4 °C. Hemoglobin solutions were at pH 6.5.

and thus, a drop in the heme peak should indicate some loss of CO even though the maximum wavelength was maintained.

Autoxidation of Oxy-Hb (Hb-Fe^{2+}−O_2) is believed to involve a nucleophilic displacement of $O_2^{\bullet-}$ (superoxide anion) from the heme by H_2O or its hydroxyl ion (OH^-), which is able to enter the heme pocket, and as a consequence leads to the conversion of Fe^{2+} to Fe^{3+}, giving met-Hb (Hb-Fe^{3+}) (*21*). met-Hb can be further oxidized in the presence of H_2O_2 (which forms from $O_2^{\bullet-}$ dismutation) and even lipid hydroperoxides to two very reactive hypervalent species, perferrylhemoglobin (•Hb-Fe^{4+}═O) and ferrylhemoglobin (Hb-Fe^{4+}═O). The displace-

ment of oxygen from the heme is accelerated at low pH (i.e., as more protons are in solution) with the help of distal histidine, which normally stabilizes the bound O_2. Access to the heme crevice plays an important role in the autoxidation of oxyhemoglobin (*23*), and the distal histidine residue has been found to be important in restricting solvent access to the heme (*20*) but at the same time can assist in the nucleophilic attack at low pH (*22*). It has also been reported that heme pocket flexibility may play a role in autoxidation, with more flexibility resulting in less stability against autoxidation and more reactive Hb (*24*). It is evident from the data presented that CO leads to a significantly different Soret heme spectrum (**Figures 1** and **4**), signifying a heme environment different from that of Oxy-Hb, possibly more compact, and as a result stabilizes the iron from oxidizing. It is also known that CO binding to heme significantly strengthens the iron−proximal histidine bond in heme proteins (*20*). The CO molecule is also bound at an angle different from that of O_2 and with different interactions with distal histidine, and the reactivity of this molecule is likely different from that of O_2. It is also possible that binding of CO to Hb leads to a spatial configuration of the whole molecule different from that when it is bound to O_2. There is evidence showing that the configuration of the molecule can have an influence on the stability toward autoxidation (*22*), since interactions in the heme pocket can be different even for subtle differences in protein conformations. It is also known that subunits of hemoglobin can dissociate and as a result be substantially less stable to autoxidation (*25*). It remains to be investigated whether CO stabilizes tilapia Hb against dissociation.

Protein Stabilization. It was also of interest to see if the high stability of CO-Hb toward autoxidation could have an impact on the stability of the protein structure. Kristinsson and co-workers (*5*, *6*) reported that CO-Hb was markedly more stable to thermal denaturation than both Oxy-Hb and met-Hb (in that order) using various conformational probes. Relative protein aggregation followed by an absorbance increase at a fixed wavelength was found to be a very useful probe for stability in these studies, and for that reason it was used on the Oxy- and CO-Hb samples here. There were significant differences in the onset and extent of protein aggregation between Oxy- and CO-Hb (**Figure 5**). Little protein aggregation was seen at 4 °C until later stages of storage (>day 19) where Oxy-Hb rapidly aggregated while CO-Hb exhibited at most ∼7.1% relative aggregation throughout the storage period (**Figure 5a**). The differences between the Oxy- and CO-Hb were more evident on frozen storage (**Figure 5b**). Significant aggregation was seen as soon as day 4 for Oxy-Hb (∼57.5% relative protein aggregation), while CO-Hb had only ∼8.3% relative protein aggregation at the same time (**Figure 5b**). While Oxy-Hb continued to aggregate on frozen storage, CO-Hb had less than 20% relative protein aggregation for all except the last two time points. It is important to note here that the thawing process may have induced some structural changes in the proteins, possibly accounting for some of the differences seen in the protein stability of Oxy- vs CO-Hb.

Aggregation away from the isoelectric point of a protein is normally associated with protein denaturation. Increased aggregation has been linked to increased denaturation for many proteins including hemoglobin (*6*, *21*). This aggregation is likely mediated via mostly hydrophobic interactions between hydrophobic regions which become progressively more exposed as hemoglobin unfolds (*21*). The heme crevice (and the heme) in hemoglobin is a highly hydrophobic part of the protein (*21*) and if exposed could be one of the key areas responsible for

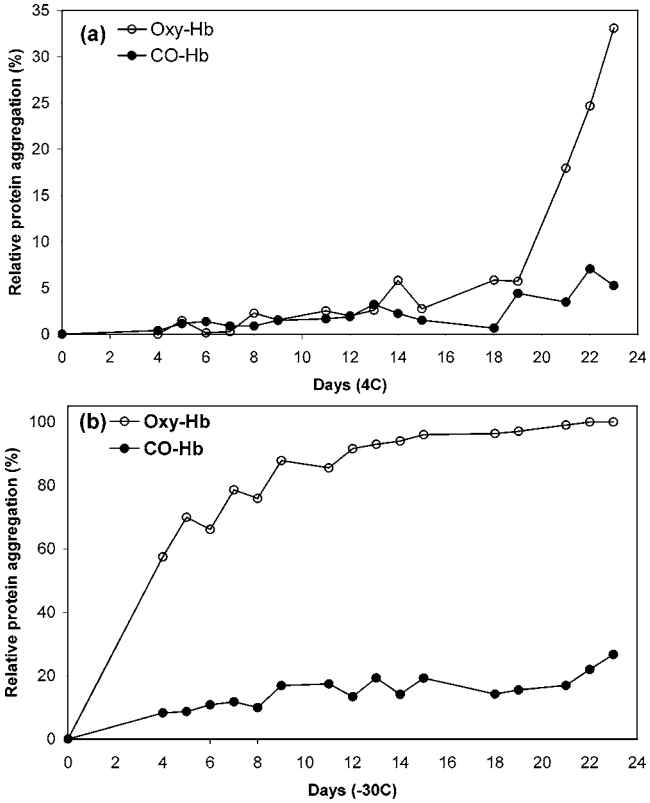

Figure 5. Aggregation of tilapia Oxy- and CO-Hb over 23 days at (**a**) 4 °C or (**b**) −30 °C. Hemoglobin solutions were at pH 6.5, and aggregation was measured spectrophotometrically.

aggregation. According to the data presented here, it is evident that CO is able to significantly stabilize the hemoglobin protein structure since aggregation was substantially less than that seen with Oxy-Hb. It is possible that this structural stabilization is related to CO's ability to stabilize the protein to autoxidation. This is supported by previously published data showing that heme peak degradation, heme peak broadening, and autoxidation were preceded by thermal aggregation of tilapia hemoglobin (*5*, *6*). It has also been found that the secondary and tertiary structures of hemoglobin were stabilized by the presence of CO (*6*). Wittung-Stafshede (*26*) has also demonstrated that oxidized heme proteins (myoglobin, cytochrome b_{562}, and cytochrome *c*) are less thermally stable than their reduced counterparts. This suggests that autoxidation destabilizes the protein, thus likely leading to progressively more exposure of the heme crevice and subsequently increased aggregation. The significant broadening of the Soret heme peak of Oxy-Hb compared to CO-Hb supports this (**Figure 4**).

Peroxidase Activity. Heme proteins have been shown to possess peroxidase activities (*16*), which means they can catalyze the oxidation of various substrates following a reaction with hydrogen peroxide (H_2O_2) (*27*). This is because H_2O_2 reacts strongly with hemoglobin and generates the very reactive ferrylhemoglobin species (*28*). It was of interest to assess the effect CO would have on the peroxidase activity of tilapia hemoglobin. Guaiacol, a methoxyphenol that the ferrylhemoglobin radical species oxidizes to a radical which can be monitored spectroscopically, was used as the substrate to assess peroxidase activity. It is evident from **Figure 6** that Oxy-Hb was able to oxidize guaiacol on hydrogen peroxide addition significantly ($p < 0.01$) faster than CO-Hb. The reaction occurred immediately on addition and within 1 s. It was also demonstrated that, in the absence of guaiacol, Oxy-Hb was significantly ($p < 0.01$) more reactive with hydrogen peroxide

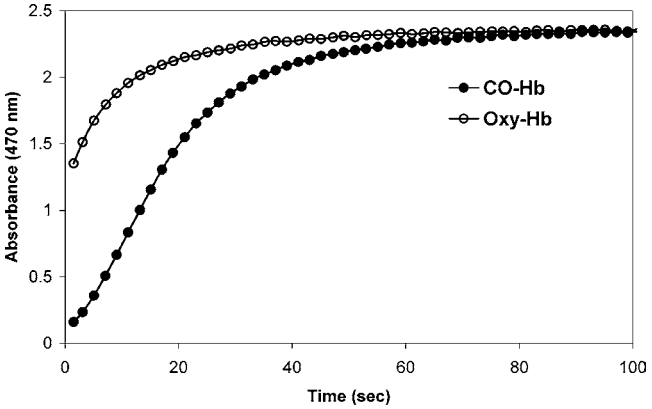

Figure 6. Peroxidase activity of tilapia Oxy- and CO-Hb at pH 6.5 using guaiacol as substrate and following the absorbance increase at 470 nm.

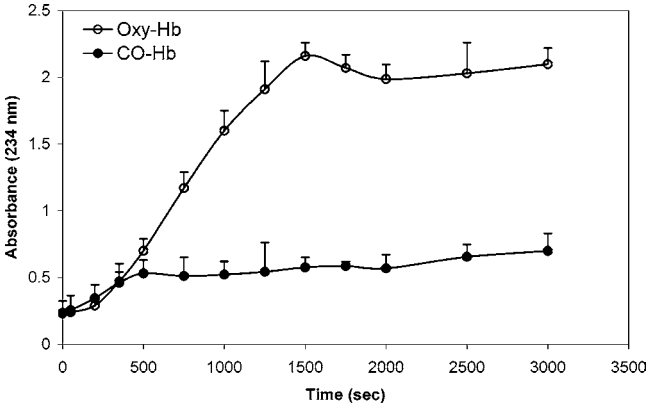

Figure 7. Pro-oxidative activity of tilapia Oxy- and CO-Hb (~2 μM) at pH 6.5 assessed by the formation of conjugated dienes at 234 nm in a linoleic acid emulsion at 4 °C.

than CO-Hb (data not shown). Fedeli and co-workers (*16*) reported that hemoglobins with higher peroxidase activity were also more susceptible to autoxidation and led to more lipid oxidation in model liposome systems. The results with Oxy- and CO-Hb presented here are in agreement with this since the hemoglobin more susceptible to autoxidation (i.e., Oxy-Hb) had significantly higher peroxidase activities. It is also possible that the lower reactivity of CO-Hb could be related to less access of guaiacol to the heme crevice. Gabbianelli and co-workers (*28*) hypothesized that this could explain the very different reactions of trout Hb I and IV with guaiacol, with the former having a more restricted access to guaiacol. As previously discussed, the Soret heme peak of CO-Hb is significantly more narrow than that of Oxy-Hb, indicating a modification in the heme pocket possibly in part limiting access of the substrate to the heme.

Pro-oxidative Activity. The mechanisms by which heme proteins catalyze or mediate lipid oxidation are still being debated. As summarized by Baron and Andersen (*29*) there is evidence that all the different heme protein species may participate in lipid oxidation. Reduced oxygenated heme proteins have been found to promote oxidation, although the mechanism is not clear. Richards and Hultin (*17*) demonstrated that the reduced deoxy form of hemoglobin mediates lipid oxidation, possibly in part because it is readily oxidized to met-Hb. There is however a strong correlation between the formation of the met form of heme proteins and lipid oxidation, especially at lower pH values and in the presence of lipid hydroperoxides (*29*). met-Hb is believed to catalyze oxidation in large part via the breakdown of preformed lipid hydroperoxides (*17*); thus, the more preformed hydroperoxides, the more effective it is as a pro-oxidant. The ferryl-Hb species that forms from met-Hb is believed by many to be the main pro-oxidative form of hemoglobin, being able to initiate lipid peroxidation. Autoxidation of Hb is thus expected to lead to more lipid oxidation. Since carbon monoxide significantly retarded the autoxidation (and peroxidase activity) of the hemoglobin molecule, it was hypothesized that lipid oxidation would be less for the model systems with CO-Hb compared to Oxy-Hb. The two systems interestingly revealed different results. In a system containing emulsified linoleic acid and CO-Hb, significantly ($p < 0.01$) less oxidation (i.e., formation of conjugated dienes) was seen compared to that in the system with Oxy-Hb (**Figure 7**). The onset of oxidation was however at similar points for both hemoglobins. Several studies with lipid emulsion model systems have demonstrated that oxidized heme proteins and heme proteins more susceptible to autoxidation are more pro-oxidative (*4*, *16*, *30*). It has also been reported that heme protein autoxidation and lipid oxidation are interrelated, since oxidation products can trigger autoxidation, which in turn gives a more reactive heme protein species (*31*). Although we could not determine the oxidation state of the hemoglobins after addition to the linoleic acid, it is expected that the increased stability of the CO-Hb toward autoxidation, and also its reduced reactivity with H_2O_2, in part accounts for the significantly suppressed pro-oxidative activity seen in the linoleic acid emulsion system. The small level of lipid oxidation seen in the system with CO-Hb could be due to a small part of the CO-Hb oxidizing in the presence of the lipid emulsion and thus resulting in a small amount of more reactive species. It is also possible that a small percentage of the hemoglobins in the CO-Hb mixture were not bound to CO (even though the spectra suggested full binding) and thus could have caused the oxidation to occur at the same point as the Oxy-Hb did, but to a significantly smaller extent (i.e., proportionally to the amount of reactive Hb). It is also possible that denaturation of Oxy-Hb in the linoleic acid/Tween emulsion system could have contributed to the higher level of oxidation, as denatured Hb would have a more open heme crevice and thus better access to the lipid substrate. Unfolding of the heme crevice can also possibly cause heme to be released into the lipid phase, thus causing even more oxidation than protein-bound heme (*21*). We have found than Oxy-Hb is more susceptible to denaturation than CO-Hb (*5*−*6*).

Interestingly, the washed tilapia and cod systems did not reveal the same striking difference between systems with CO-Hb or Oxy-Hb when they were exposed to air at 4 °C ($p > 0.05$) (**Figure 8**), while the system kept in ~100% CO showed a significantly ($p < 0.01$) suppressed lipid oxidation (**Figure 8**). Almost no oxidation was also seen in the system without added Hb. The low level of oxidation in an environment almost saturated with CO was not unexpected since little oxygen is available for oxidation. Work with dark and white muscle from Spanish mackerel (*8*) and mahi mahi (*10*) has shown that oxidation was significantly suppressed when fillets were kept in a ~100% CO environment for 8 days compared to when fillets were treated with 100% CO for 24 h and then exposed to air for 7 days (*8*). That study did however show that development of oxidation did still occur in 100% CO environments although it occurs slower than in air, possibly due to breakdown of preformed hydroperoxides and/or residual oxygen in the fish muscle. The same study showed a slight suppression in oxidation when fillets were treated with 100% CO for 24 h and then exposed to air compared to when fillets were untreated. Fukunaga and co-workers (*32*) reported that red blood cells from trout treated with CO were more resistant to membrane lipid

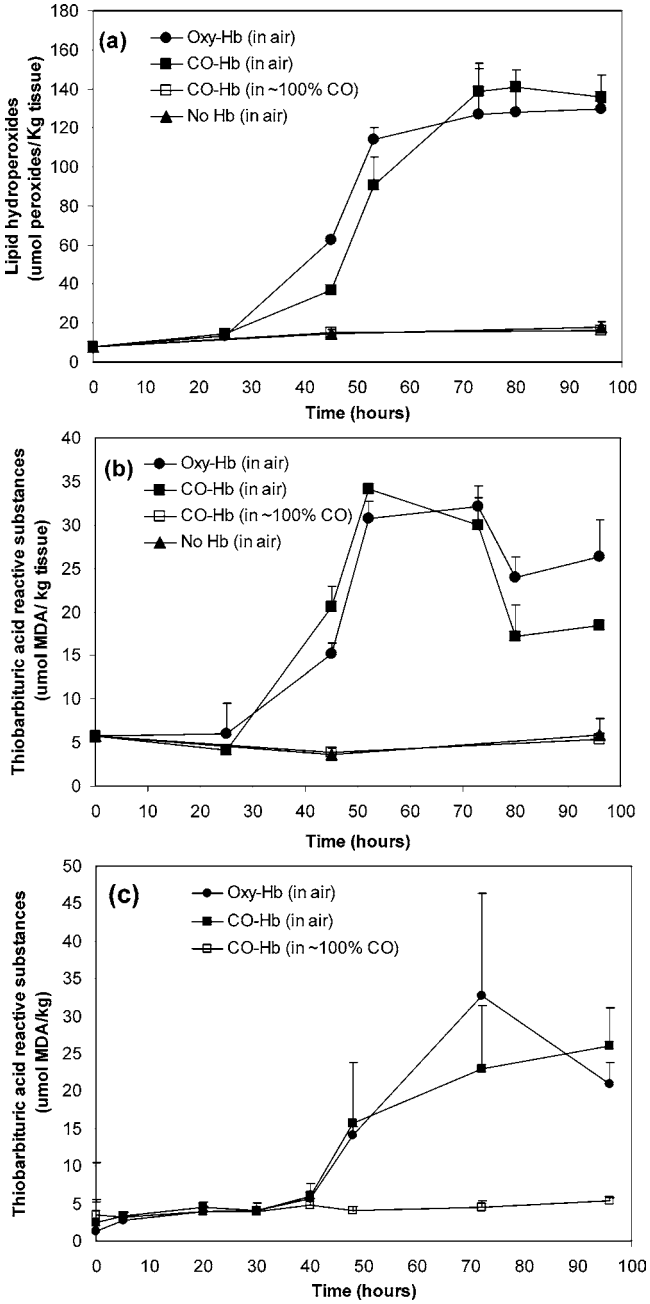

Figure 8. Pro-oxidative activity of tilapia Oxy- and CO-Hb at pH 6.5 in washed tilapia and cod white muscle systems at 4 °C: (**a**) formation of lipid hydroperoxides in a washed tilapia white muscle system, (**b**) formation of TBARS in a washed tilapia white muscle system, (**c**) formation of TBARS in a washed cod white muscle system. Hemoglobin was added (~6 μmol/kg of tissue) to the washed systems which were at ~86% moisture, and they were kept in Petri dishes (5 mm layer) with the lid on at 4 °C. Tissue plugs were sampled at regular intervals for analysis.

oxidation on ozone treatment than normal red blood cells. The above suggests that CO-Hb/Mb might be less pro-oxidative than Oxy-Hb, which would agree with the results seen for the linoleic acid emulsion.

The onset of lipid oxidation occurred at similar times in the air-exposed washed tilapia muscle systems containing CO- and Oxy-Hb (**Figure 8**). The system with Oxy-Hb did however develop lipid hydroperoxides sooner than the system with CO-Hb (**Figure 8a**), while the opposite was seen for the TBARS results (**Figure 8b**). The pro-oxidative activity of tilapia Hb was also evaluated in a washed cod system (**Figure 8c**). These results show that the system with Oxy-Hb reached peak TBARS formation sooner than the system with CO-Hb; however, the difference was not significant ($p > 0.05$). These studies therefore suggest that there was little difference between the two Hb derivatives in the washed fish systems. It was expected that the system with CO-Hb would be less pro-oxidative since the CO-Hb was found to have much greater stability against autoxidation and thus would have been maintained in the reduced state longer than Oxy-Hb. There is however evidence in the literature that reduced hemoglobins may also have significant pro-oxidative activity (*29*). Why the CO-Hb and Oxy-Hb yielded similar oxidation development in the washed cod system is interesting and is under investigation. Both CO- and Oxy-Hb are in their reduced state, while CO binding maintains the CO-Hb in a reduce state for substantially longer periods (as indicated by autoxidative results). It is possible that the tilapia Hb's oxidized the lipids in the washed cod system in their reduced state, thus accounting for the similar results. It is also possible that on diluting the Hb into the washed muscle it could have dissociated. Dissociated Hb is more susceptible to autoxidation (*25*), and thus, both derivatives could have rapidly oxidized to the met-Hb form, which then would have triggered oxidation at similar time points for both species. Dilution of the CO-Hb into the washed muscle could also have destabilized the CO binding to the heme and thus led to a rapid release of CO from Hb, and therefore, at the onset of oxidation (~25 h) similar levels of met-Hb could have been formed for both systems. However, these are all speculations which need further investigation to elucidate the pro-oxidative differences between the two binding states of hemoglobin. The wide application of CO gas treatment in the aquatic foods industry makes it very important to understand how these processes work on the molecular level and how they influence product quality.

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