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Efficiency of Hemoglobin from Rainbow Trout, Cod, and Herring in Promotion of Hydroperoxide-Derived Free Radicals

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Hemoglobin (Hb) from rainbow trout (*Oncorhynchus mykiss*), Atlantic cod (*Gadus morhua*), and herring (*Clupea harengus*) were evaluated in terms of capacity to generate hydroperoxide-derived free radicals in aqueous solution at pH 6.8. Cumene hydroperoxide (CumOOH) was used as a model for preformed lipid hydroperoxides, and free radicals were monitored by stabilizing with the spin trap α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) and further detection by electron spin resonance spectroscopy. The generation of hydroperoxide-derived free radicals was found to be Hb concentration-dependent, and intermediate Hb concentrations corresponding to hydroperoxide/Hb molar ratios of roughly 200 were found most active. The results indicated the following relative activity in the formation of hydroperoxide-derived free radicals under low and medium hydroperoxide levels (hydroperoxide/Hb molar ratios ≤5): cod Hb ≈ herring Hb > trout Hb. This free radical generating-activity was not affected by heating fish hemoglobins at 70 °C for 10 and 45 min in aqueous solution. Cod and herring Hb also exhibited faster autoxidation rates and stronger redox instability in the presence of hydroperoxides compared to trout Hb. According to their ability to generate hydroperoxide-derived free radicals, hemoglobin from cod and herring showed more activity than trout Hb as catalysts of lipid oxidation in lecithin liposomes. These data emphasize the central contribution of the free radicals formed through hydroperoxide-decomposition on the hemoglobin-mediated lipid oxidation.

KEYWORDS: Lipid oxidation; fish hemoglobin; lipid hydroperoxides; free radicals; ESR; spin-trapping

INTRODUCTION

Lipid oxidation during processing and storage is a principal cause of quality deterioration and shelf life shortening of fish-based food (*1*). Fish muscle is characterized by a high proportion of polyunsaturated fatty acids (PUFA) that are extremely vulnerable to oxidative degradation. Among fish species, the development of lipid oxidation is extremely rapid for pelagic fish given that their muscle holds an important residual amount of hemoglobin (Hb) and/or myoglobin (Mb), and that such heme pigments have been found to catalyze lipid oxidation extensively in isolated fish membranes (*2*, *3*) and washed fish muscle (*2*, *4*), devoid of residual heme pigments.

Different treatments or combinations of such treatments have been attempted in order to diminish either the content or pro-oxidative activity of Hb/Mb in fish muscle. Technological procedures based on bleeding (*4*) and washing with water (*5*, *6*) have been employed to remove catalytic Hb from fish muscle; however, the efficiency of such treatments to delay lipid oxidation has been found to be strongly dependent on the physical state of the raw material (intact, filleted, or minced fish), freshness, and even on the fish species. The combined effect of a washing step with exogenous antioxidant supplementation has frequently been demonstrated to be more efficient in preventing lipid oxidation in fish muscle (*7*, *8*). The incorporation of press juice from fish muscle (*9*, *10*) or of several phenolic compounds, including grape procyanidins (*8*), hydroxytyrosol (*8*, *11*), caffeic acid (*12*), and cranberry components (*13*), have been shown to efficiently inhibit the development of lipid oxidation in washed fish muscle triggered by fish hemoglobin or spontaneously in pelagic fish muscle.

Hemoglobins have the ability to decompose preformed lipid hydroperoxides, in effect generating free radicals in what has been postulated to be an important mechanism in the pro-oxidative behavior of hemoglobins (*14*). Alkoxyl radicals may subsequently propagate lipid peroxidation in the presence of oxygen through a radical chain mechanism. Several investigations have reported varying efficiencies in promoting lipid oxidation among hemoglobins from different fish species, e.g., pollock Hb is more pro-oxidant in washed fish muscle at pH 7.2 than Hb from mackerel, followed in decreasing order by menhaden Hb and flounder Hb (*15*). Pollock Hb has also been found to have higher activity in promoting lipid oxidation compared to that of horse mackerel Hb, which on the other hand was found to be more effective than seabass Hb (*16*). Trout Hb exhibits likewise greater pro-oxidant ability than Hb from tilapia, a warm-water fish, whereas hemoglobin from the pelagic species mackerel and herring is more

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active than trout Hb (*17*, *18*). However, there is no information available about the specific ability of fish hemoglobins to promote the generation of hydroperoxide-derived free radicals and the individual contribution of this pathway in the pro-oxidant behavior of fish hemoglobin.

The present investigation was accordingly aimed to gain insight into the capacity of fish hemoglobin to generate free radicals in the presence of preformed lipid hydroperoxides. Hemoglobin from Rainbow trout (*Oncorhynchus mykiss*), Atlantic cod (*Gadus morhua*), and herring (*Clupea harengus*) was evaluated in terms of capability in generating free radicals by using cumene hydroperoxide (CumOOH) as a model for lipid hydroperoxides and monitoring the formation of free radicals by electron spin resonance (ESR) spectroscopy. In order to provoke a weakening of the heme−globin bond and to study any influence on the generation of hydroperoxide-derived free radicals, fish hemoglobins subjected to thermal treatments were also evaluated. The capacity of fish hemoglobins to generate hydroperoxide-derived free radicals was further related to their redox stability in the absence (autoxidation) or the presence of hydroperoxides and with their capacity to promote lipid oxidation in liposomes.

MATERIALS AND METHODS

Materials. Rainbow trout (*Oncorhynchus mykiss*) and Atlantic cod (*Gadus morhua*) were provided alive by local fish suppliers in Denmark. Herring (*Clupea harengus*) was obtained in rigor mortis state from a local fish market in Copenhagen. α-(4-Pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN), cumene hydroperoxide (CumOOH), sodium heparin, tris-[hydroxymethyl]aminomethane (Tris), bovine hemoglobin, ammonium persulfate, sodium dithionate, dibasic sodium phosphate ($Na_2HPO_4 \cdot 2H_2O$), monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$), and lecithin from soybean with a 30% of L-α-phosphatidylcholine were purchased from Sigma (Steinheim, Germany). Shephadex G-25 columns were obtained from GE Healthcare (Uppsala, Sweden). All chemicals were of analytical grade, and water was distilled and further deionized using a Milli-Q system (Millipore, Billerica, Massachusetts).

Isolation of Fish Hb. Eight animals were employed to obtain a pool of rainbow trout or Atlantic cod Hb, and 40 individuals were used for herring Hb. Blood was collected with a transfer glass pipet rinsed with 150 mM NaCl and sodium heparin solution (30 units/mL) from the caudal vein after cutting off the tail of individual fish in the rigor state. Blood was immediately mixed with approximately one volume of the saline sodium heparin solution. The hemolysate was then prepared according to the modifications by Richards and Hultin (*4*) of the Fyhn's et al. procedure (*19*). The visible spectrum of fish Hb was acquired in a Cary 100 UV−vis spectrophotometer (Varian, Palo Alto, CA) and showed the appearance of two absorption peaks at 540 and 570 mn that are characteristic for oxygenated Hb (oxyHb). Hb was stored at −80 °C and was thawed just before use.

Preparation of Methemoglobin (MetHb) and Heated Hb. MetHb from Rainbow trout Hb was obtained by modification of the Svistunenko's et al. procedure (*20*). Briefly, Hb was incubated with 4 mM ammonium persulfate for 1 h at room temperature and then passed down through a Shephadex G-25 column. MetHb was stored at −80 °C and was thawed just before use.

Heated Hb was prepared by heating 300 μL of a 500 μM Hb solution in a closed Eppendorf tube. Hb solutions were subjected to two different thermal treatments in a water bath by heating at 70 °C for 10 or 45 min. Heated Hb was prepared daily and vigorously stirred just prior to transfer for reaction with hydroperoxides.

Quantification of Fish Hb. Hemoglobin was quantified either in the hemolysate directly obtained from fish blood or in the metHb solution according to Brown (*21*). Briefly, Hb diluted in 50 mM Tris, pH 8.6, was mixed with around 1 mg of sodium dithionite and bubbled with carbon monoxide gas for 20 s. The sample was then scanned from 400 to 480 nm against a blank that contained only buffer. A standard curve was made by relating the peak absorbance (≈ 416 nm) minus the absorbance at the valley (465 nm) with known concentrations of bovine hemoglobin.

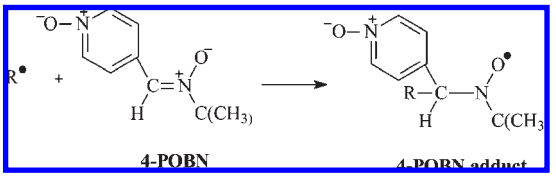

Figure 1. Formation of long-lived nitroxyl radical spin adducts by the addition of free radicals to the nitrone spin trap 4-POBN.

Autoxidation of Fish Hb. The spontaneous formation of metHb was studied in 50 mM phosphate buffer (pH 6.8) at 4 °C. Solutions contained 20 μM of the corresponding fish Hb. MetHb formation was calculated at different incubation times by adaptation of Winterbourn's equation that estimates the concentration of metHb at micromolar levels (on a hemoglobin basis) considering the absorbance at 576 and 630 nm (*22*):

$$[MetHb] = \left(\frac{279A_{630} - 3A_{576}}{4}\right)$$

Redox Stability of Fish Hb in the Presence of Cumene Hydroperoxides (CumOOH). The different fish Hbs were exposed to CumOOH in 50 mM phosphate buffer, pH 6.8. The final concentration of Hb and CumOOH was 10 and 200 μM, respectively. The visible spectra were then monitored in the range 500−650 nm after 1, 3, and 6 min of the Hb/CumOOH interaction and compared with the corresponding fish Hb not exposed to CumOOH.

ESR Spin-Trapping Experiments. A solution of the spin trap POBN was prepared daily in 50 mM phosphate buffer, pH 6.8. Fish Hb was incorporated into the above spin trap solution, and the reaction was then initiated by the addition of cumene hydroperoxide (CumOOH) in ethanol as previously described (*23*). Ethanol constituted approximately 2.5% of the reaction mixture. POBN was present in a final concentration of 60 mM. Fish Hb was tested in concentrations ranging 0.5−134 μM (on hemoglobin basis), and CumOOH was employed at the concentration range 23−11400 μM. The reaction mixture was immediately subjected to vigorous stirring and transferred to a 50 μL disposable glass capillary (Magnettech, Germany). ESR spectra were recorded at room temperature on a Miniscope MS200 spectrometer (Magnettech, Germany) using the following ESR-settings: microwave power, 9.4 GHz; sweep width, 70 G; sweep time, 60 s; modulation amplitude, 2 G; time constant, 0.1 s. Peak-to-peak amplitudes were used for the quantification of the intensity signal of POBN-spin adducts (**Figure 1**). Simulation and fitting of ESR spectra were performed by the WinSIM program (*24*).

Experiments in Liposomes. Liposomes were prepared daily by adaptation of the procedure described by Huang and Frankel (*25*). Briefly, lecithin at a concentration of 0.8% (w/w) was dissolved in 50 mM phosphate buffer, pH 6.8, by magnetic stirring and subsequently sonicated for 10 min with a water-bath sonicator. Liposomes were then filtered through paper filter, introduced into 50 mL Erlenmeyer flasks, and incubated at 30 °C in a thermostatted water-bath. Liposomes were supplemented with 5 μM hemoglobin solution, and lipid oxidation was evaluated by monitoring conjugated dienes spectrophotometrically with time.

Conjugated Dienes. Liposomes (100 mg) were dissolved in 5 mL of methanol, and the absorbance was measured at 234 nm (UV−vis spectrophotometer; Perkin-Elmer). Conjugated dienes were expressed as mmol hydroperoxides/kg phosphatidylcholine (PC) as previously described by Frankel et al. (*26*).

Statistical Analysis. The experiments were performed at least twice, and data are reported as the mean ± standard deviation of three replicates ($n = 3$). The data were analyzed by one-way analysis of variance (ANOVA) and the least-squares difference method. Differences were considered as statistically significant for a confidential interval of 95% ($p < 0.05$). Statistical analyses were performed with the software Statistica 6.0.

RESULTS AND DISCUSION

Effect of Hb-Concentration on the Generation of CumOOH-Derived Free Radicals. The spin trap POBN was used to stabilize

the hydroperoxide-derived free radicals by forming long-lived radical spin adducts, as illustrated in **Figure 1**. ESR spectra consisted of a triplet of doublets, which was successfully simulated by a POBN-spin adduct with coupling constants $a_N = 15.4$ G and $a_H = 2.4$ G, and assigned to a POBN-carbon centered radical adduct, as previously reported (*23*). Under the current experimental conditions, the formation of POBN-radical adducts was essentially heme-mediated and not attributed to the free inorganic iron coming from the Hb disintegration given that the addition of the iron-chelating agent EDTA did not significantly affect the formation of the spin adduct (*23*). It is in agreement with a previous investigation in which it was found that hydroperoxide decomposition in fish membranes triggered by horse mackerel Hb was much faster than hydroperoxide decomposition activated by the ferrous ion (*3*). Additionally, we have previously reported an extensive reduction in the formation of carbon-centered radicals through ferrous-catalyzed decomposition of CumOOH by increasing phosphate concentration up to 50 mM, as used in the present experiments (*27*).

The capacity of different concentrations of trout and cod Hb ranging from 0.5 to 134.0 μM to promote the formation of free radicals was initially evaluated in the presence of 1140 μM CumOOH (hydroperoxide/Hb ratio: 8.5−2280) (**Figure 2**). After 1 min of reaction, the three most elevated concentrations of trout Hb (27.9, 57.1, and 134.0 μM) originated the most significant generation of radicals ($p < 0.05$). However, a stronger accumulation of POBN-radical adducts was seen for an intermediate trout Hb concentration ranging from 4.6 to 22.9 μM after 6 min of reaction. This observation is the consequence of a much higher formation rate of the POBN-radical adducts between 1 and 6 min for 4.6 or 22.9 μM in comparison with the other Hb concentrations investigated. A relevant characteristic was that the highest trout Hb concentration was found to be the only concentration without any net formation of free radicals between 1 and 6 min, whereas a continuous accumulation of free radicals was observed for the other Hb concentrations investigated. A similar concentration-dependent behavior was observed for cod Hb, and accordingly, the intermediate concentrations 4.6−22.9 μM were found to be more active to promote the formation of POBN-spin adducts during the interaction of cod Hb with 1140 μM CumOOH.

The concentration-dependence for trout Hb was also investigated with a CumOOH concentration 10-fold higher (11400 μM), and the results revealed a gradual increase up to approximately 57 μM of the optimum Hb concentration for formation of free radicals (**Figure 2**). In sum, the generation of hydroperoxide-derived free radicals was found to be Hb concentration-dependent, and therefore, Hb is concluded to function not only as a catalyst but also as a reactant. This conclusion is in agreement with the results of previous investigations showing that a rising concentration of fish Hb increases both the rate and the extent of lipid oxidation in washed fish muscle (*4*) and in fish membranes (*2*).

Capacity of Rainbow Trout, Atlantic Cod, and Herring Hb to Generate Free Radicals in the Presence of Hydroperoxides. The efficiency of trout, cod, and herring Hb to promote the generation of hydroperoxide-derived free radicals was evaluated for three different CumOOH concentrations (22.9, 114, and 571 μM), with a Hb concentration of 22.9 μM. For equimolar conditions of Hb and CumOOH (22.9 μM), Hb from cod and herring displayed greater capacity to generate POBN-radical adducts than trout Hb (**Figure 3**). For trout Hb, free radicals were not detected after 1 min of interaction with CumOOH, in contrast to cod and herring Hb that both produced detectable amounts of free radicals. The formation of POBN-spin adducts increased with time for all fish

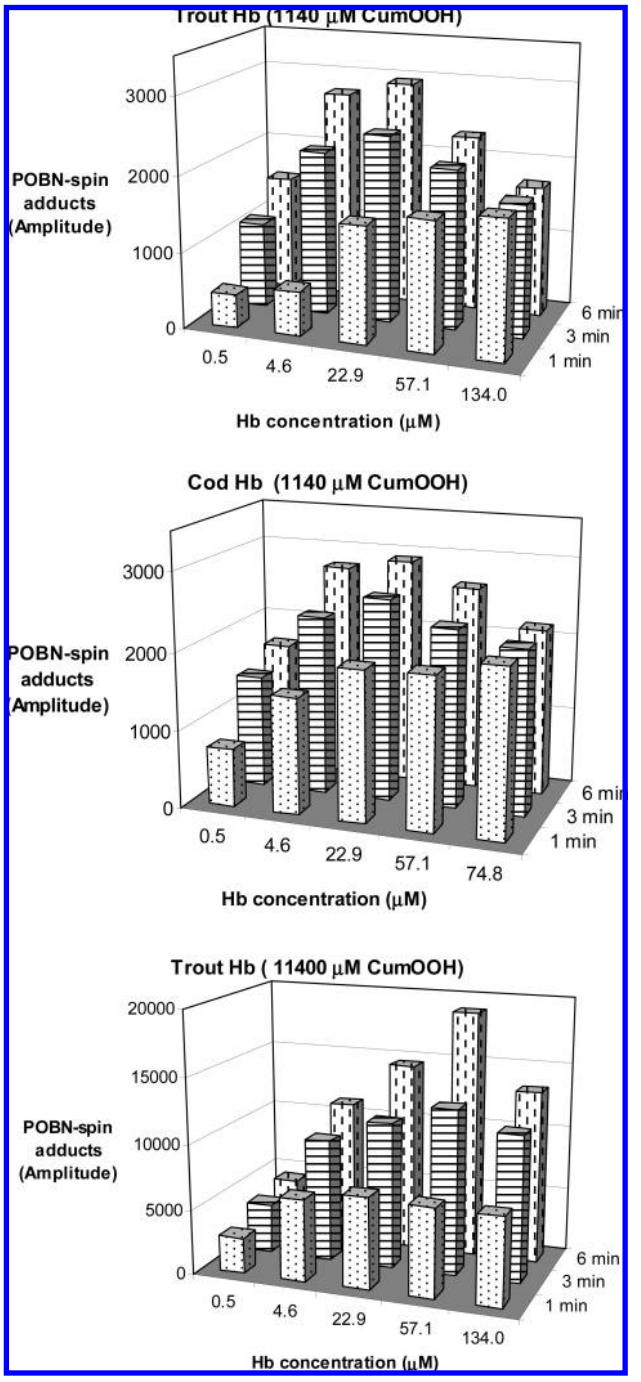

Figure 2. Effect of Hb concentration on the generation of POBN-spin adducts. The formation of POBN-spin adducts was evaluated in a broad range concentration for rainbow trout or Atlantic cod Hb (0.5−134.0 μM) under two different levels of cumene hydroperoxide (CumOOH), 1140 and 11400 μM. The spin trap POBN was added in a final concentration of 60 mM, and the reaction medium was 50 mM phosphate buffer, pH 6.8.

hemoglobin tested, but still those from cod and herring produced a higher level of free radicals after longer reaction times (6−9 min) ($p < 0.05$). Consequently, hemoglobin from cod and herring was found to be more active in catalyzing the formation of hydroperoxide-derived free radicals compared to trout Hb in environments with hydroperoxide/Hb molar ratios close to 1 or lower (data not shown). The increase of this ratio to approximately 5 by raising the CumOOH concentration to 114 μM, revealed a similar relative free radical generating activity: cod Hb ≈ herring Hb > trout Hb. Trout Hb provoked lower amounts of POBN-radical

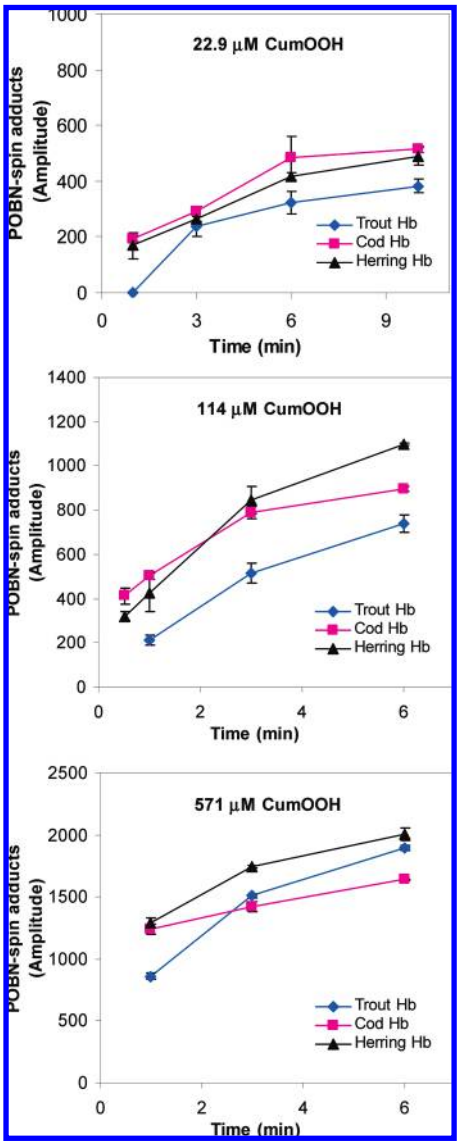

Figure 3. Comparison of rainbow trout, Atlantic cod, and herring Hb as promoters of POBN-radical adducts in the presence of different concentrations of CumOOH: 22.9 μM, 114 μM, and 571 μM. Fish hemoglobins were tested at 22.9 μM in 50 mM phosphate buffer, pH 6.8, containing 60 mM of the spin trap POBN.

adducts to be formed compared to cod and herring Hb, which exhibited similar activity during all incubation times tested except for 6 min (**Figure 3**). Upon increasing the hydroperoxide concentration to 571 μM to reach a hydroperoxide/Hb molar ratio of 25, trout Hb became significantly ($p < 0.05$) less effective than cod HB and herring HB in promoting POBN-radical adducts after 1 min. Cod and herring Hbs showed similar activity at the same incubation time in agreement with the above results for low hydroperoxide levels. After longer incubation times, trout Hb enhanced the capacity to promote hydroperoxides-derived free radicals, resulting in activity rather similar to that found for herring Hb (**Figure 3**).

Efficiency of Heated Hb in Generating Hydroperoxide-Derived Free Radicals. The experiments were planned considering that heating is widely used during cooking and industrial processing and that heating as a thermal process unfolds the globin of heme proteins, resulting in denaturation of the globin. Unfolding of deoxymyoglobin has been found to disrupt the heme-proximal histidine bond, facilitating the release of hemin from the globin (*28*). The activity of the heated and unheated Hb was compared in order to identify changes in the capacity to generate radicals as consequence of the thermal process. Hemoglobins were subjected to two different thermal treatments, a milder treatment of heating for 10 min at 70 °C, and more severe heating for 45 min at the same temperature. Results did not show significant differences in the activity of fish hemoglobin before and after heating (**Figure 4**). Trout Hb subjected to heating during 10 or 45 min resulted in the formation of free radicals at levels similar to that for unheated trout Hb (**Figure 4A**). Heated and unheated trout Hb both exhibited increasing rate of formation of POBN-radical adducts. Identical trends were observed for cod and herring Hb (**Figure 4B** and **4C**). Kristensen and Andersen (*29*) have observed an increment in the pro-oxidative character of metMb in linoleic acid emulsions at temperatures immediately below the denaturation temperature of metMb, which was attributed to the weakening of the hemin−globin linkage, whereas higher temperatures extensively decreased its pro-oxidative activity. Bou et al. (*30*) have also reported stronger pro-oxidant activity of metMb subjected to temperatures ranging from 45 to 75 °C, in comparison with that of native metMb and metMb heated at 85 and 95 °C. In contrast, oxygenated Mb resulted in less pro-oxidative activity after heating at 45−95 °C. The reduced capacity to promote lipid oxidation at higher temperatures has been ascribed to its loss of solubility and/or formation of hemicromes, in which an additional nitrogen-based covalent bond is established between the heme group and globin, in effect decreasing hemin release.

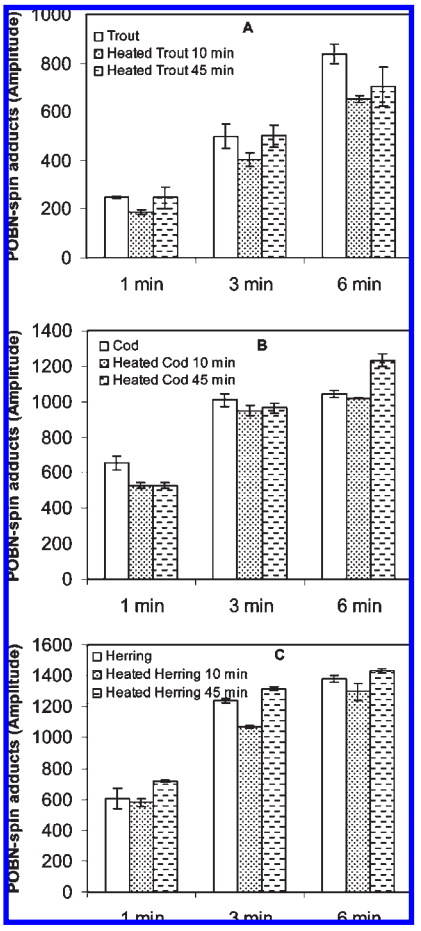

Figure 4. Comparative effect of native and heated Hb from rainbow trout (**A**), Atlantic cod (**B**), and herring (**C**) on the generation of POBN-radical adducts. Fish hemoglobins were tested native (not heated) and after heating at 70 °C for 10 or 45 min.

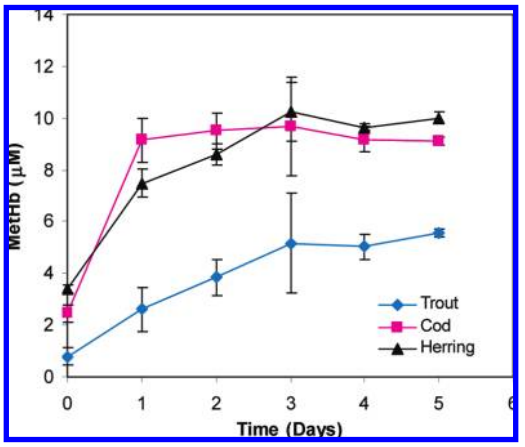

Figure 5. Autoxidation rates for rainbow trout, Atlantic cod, and herring Hb. Fish hemoglobins at 20 μM were incubated in 50 mM phosphate buffer, pH 6.8, at 4 °C.

Autoxidation of Fish Hb. The Hb isolated from trout at a concentration of 20 μM had an initial metHb level of around 0.8 μM in 50 mM phosphate buffer, pH 6.8, and this initial metHb concentration was significantly lower than those found for cod and herring Hb, this ranging between approximately 2.5−3.5 μM (**Figure 5**). Cod and herring Hb exhibited greater formation of metHb during the first day of incubation at 4 °C, and the observed increase of metHb was equal to 4.0−6.5 μM. The quantity of metHb for those Hbs was barely increased after 2 days of incubation. Conversely, trout Hb continued to accumulate metHb during the initial 3 days of incubation and to maintain the metHb levels during the following days. The accumulation of metHb was significantly lower for trout Hb during the entire experiment, while significant differences were not detected between cod and herring Hb. In summary, the autoxidation rates showed the following increasing order: Cod Hb ≈ herring Hb > trout Hb.

The results suggest a positive correlation between the susceptibility of fish Hb to undergo spontaneous oxidation to metHb and their activity in generating hydroperoxide-derived free radicals (cod Hb ≈ herring Hb > trout Hb). Taking into consideration that metHb has not demonstrated stronger capacity than oxyHb to promote the formation of hydroperoxide-derived free radicals (*23*), the presence of different levels of metHb species cannot directly explain the radical-generating activities observed for fish Hbs. However, the stronger free radical generating capacity of a specific fish Hb appears to be correlated with a higher accessibility of water/hydroperoxides to the heme crevice, which also is modulating the realtive rate of Hb autoxidation. A preceding investigation has revealed that the accessibility to the heme group in particular improves the free radical generating capacity in the presence of low hydroperoxide concentrations since the decreasing order of activity was found to be free hemin (ferric form of heme group) > hemin/BSA > trout Hb (*23*). However, high hydroperoxide concentrations or successive interactions with hydroperoxides significantly reduced the free radical generating activity of the heme-containing systems with high accessibility to the heme group. This could be caused by a more intense heme-decomposition by the generated radicals when the heme group is highly accessible.

Redox Stability of Fish Hb in the Presence of CumOOH. The destabilizing effects of hydroperoxides on trout, cod, and herring Hb were monitored by following their spectral changes in the range 500−650 nm, the spectral region in which the different redox forms of Hb (oxyHb, deoxyHb, metHb, and ferrylHb)

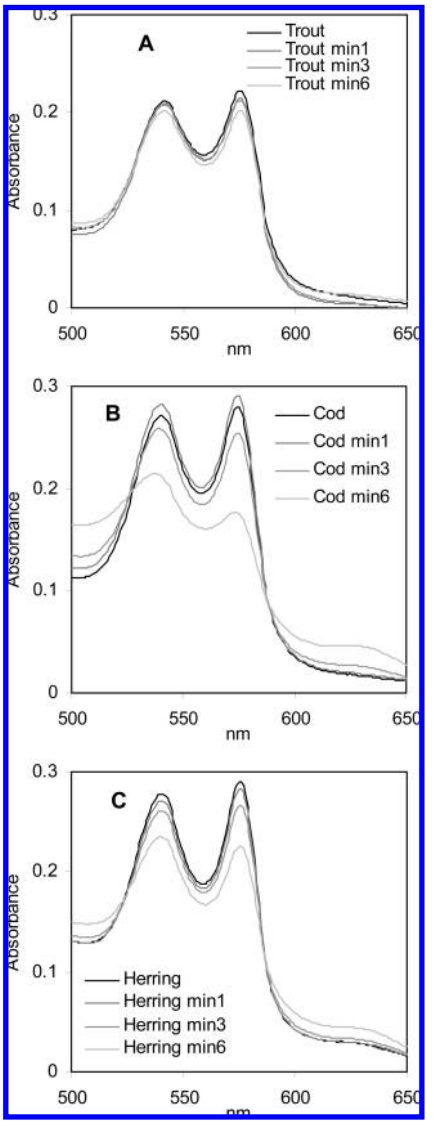

Figure 6. Spectral changes for rainbow trout (**A**), Atlantic cod (**B**), and herring Hb (**C**) during the incubation with CumOOH in 50 mM phosphate buffer, pH 6.8. Final concentrations of Hb and CumOOH were 10 and 200 μM, respectively.

possess characteristic absorption spectra (*22*). The results showed less spectral modifications for trout Hb than for Hb from cod and herring (**Figure 6**). The highly oxygenated Hb (oxyHb) from trout suffered only minor changes during 6 min of incubation with CumOOH. In contrast, hemoglobins from cod and herring were found to be less stable, considering the important loss in intensity of the absorbance peaks at 540 and 576 nm, which are characteristic for oxyHb. Additionally, the increased absorbance at 630 nm for cod and herring Hb indicated that oxyHb is at least partially converted to metHb by interaction with hydroperoxides. The present results indicated the following redox instability in the presence of preformed hydroperoxides: cod Hb ≈ herring Hb > trout Hb. Consequently, there is a positive correlation between the capacity of fish hemoglobin to generate hydroperoxide-derived free radicals (cod Hb ≈ herring Hb > trout Hb) and their redox instability in the presence of CumOOH. Two principal features may explain the direct relationship between the redox instability of fish Hb and their free radical generating capacity: (i) most of the mechanisms postulated for the production of free radicals by oxyHb involve a simultaneous oxidation to metHb or even to ferrylHb (*31*, *32*), and (ii) oxyHb could be directly

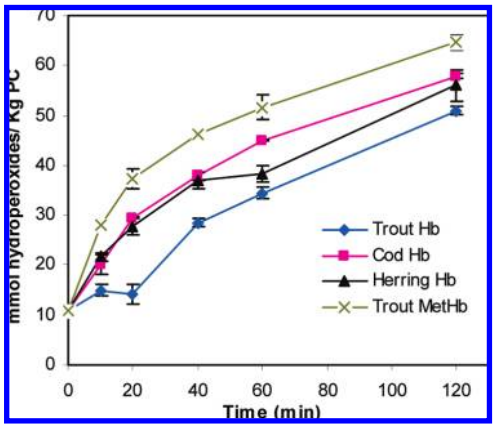

Figure 7. Pro-oxidant activity of hemoglobin from rainbow trout, Atlantic cod, and herring, and trout metHb in liposomes. Liposomes were prepared in 50 mM phosphate buffer, pH 6.8, and supplemented with the corresponding fish Hb at 5 μM. Conjugated dienes were monitored during incubation at 30 °C.

oxidized by hydroperoxide-derived free radicals, and therefore, an environment with higher amounts of free radicals should diminish the redox stability of Hb.

Pro-Oxidant Capacity of Rainbow Trout, Atlantic Cod, and Herring Hb in Liposomes. The ability of fish Hb to initiate and catalyze lipid oxidation was evaluated in liposomes incubated at 30 °C. The formation of conjugated dienes revealed a slower initiation and development of lipid oxidation for trout Hb compared to those of cod and herring Hb (**Figure 7**). In liposomes supplemented with trout Hb, the formation of conjugated dienes exhibited an induction period of 20 min for the actual experimental conditions, while conjugated dienes were found to increase from the time of Hb addition in the case of cod and herring Hb without a lag phase. The levels of conjugated dienes were significantly lower for trout Hb during the entire incubation period, whereas cod and herring Hb showed a similar pro-oxidative activity except for the monitoring time of 60 min. The activity of trout Hb, which was mainly present as oxyHb, was also compared with trout metHb. The results indicated a more intensive activation of lipid oxidation by trout metHb compared to that by trout Hb, which is in concordance with previous investigations that revealed stronger pro-oxidative capacity for metHb compared to that of the corresponding reduced Hb species (*16*, *33*). Trout metHb was also more active in promoting lipid oxidation than cod and herring Hb, both which of had a stronger pro-oxidative capacity than trout Hb. In summary, the relative activity in promoting lipid oxidation in liposomes was found to be trout metHb > cod Hb ≈ herring Hb > trout Hb. This pro-oxidative effectiveness of fish hemoglobin in liposomes is seen to be similar to that observed for the free radical-generating capacity in the presence of model hydroperoxides. Therefore, the pro-oxidant activity of fish Hb appears to be explained at least in part by its ability to promote free radicals through the decomposition of preformed lipid hydroperoxides. However, the strong susceptibility of cod and herring Hb to be oxidized to metHb in both spontaneous and hydroperoxide-mediated conditions should also have an important contribution to their strong pro-oxidant behavior since our results also revealed that the metHb form is a more active initiator of lipid oxidation than the corresponding oxyHb form (**Figure 7**). Taking into account that metHb and reduced Hb species demonstrated similar ability to promote hydroperoxide-derived free radicals (*23*), a reliable explanation for the superior pro-oxidant activity of metHb compared with that of oxyHb could be that metHb provides a higher production of ferrylHb (*31*), which is able to initiate lipid oxidation by abstraction of a hydrogen atom from any PUFA.

To summarize, the present study has revealed noteworthy differences among fish Hbs in the ability to promote free radicals in the presence of preformed hydroperoxides. The generative capacity of hydroperoxide-derived free radicals seems to display a direct correlation with the hemoglobin vulnerability to undergo oxidative alterations either in spontaneous or hydroperoxide-forced conditions: cod Hb ≈ herring Hb > trout Hb. This observation is reasonable taking into consideration that the heme group is the active site for both processes, and therefore, the accessibility by water/hydroperoxides to the heme crevice should be a limiting factor for the generation of hydroperoxide-derived free radicals by hemoglobins. Cod and herring Hb exhibited more activity than trout Hb as an initiator of lipid oxidation in liposomes, according to their ability to generate free radicals in the presence of preformed hydroperoxides. These data emphasize the central contribution of the free radicals generated through hydroperoxide-decomposition to hemoglobin-catalyzed lipid oxidation.

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