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Potential of Peroxynitrite To Alter the Color of Myoglobin in Muscle Foods

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Superoxide anion and nitric oxide can react to form the highly oxidizing species peroxynitrite. The objective of this research was to determine if peroxynitrite can promote the discoloration of myoglobin under conditions expected in muscle foods. Reagent peroxynitrite (25−100 μM) caused rapid and extensive formation of metmyoglobin from oxymyoglobin with the majority of metmyoglobin formation occurring during the first 5−10 min of incubation. Carbon dioxide caused a small decrease in the ability of peroxynitrite to oxidize oxymyoglobin, and peroxynitrite-promoted conversion of oxymyoglobin to metmyoglobin increased with decreasing pH (5.5−7.0). Differential scanning calorimetry suggested that peroxynitrite caused minimal changes in myoglobin structure. These results indicate that peroxynitrite can promote the conversion of oxymyoglobin to metmyoglobin under the conditions expected in muscle foods.

KEYWORDS: Myoglobin; meat color; peroxynitrite; nitric oxide; metmyoglobin

INTRODUCTION

The main sensory property consumers use to judge the quality of retail meat is color (*1*). The desirable red color in meat is produced by myoglobin, a heme-containing protein, the molecular state of which will ultimately determine the color and salability of the product. The oxidation state of the iron at the center of the heme group and what is bound to its sixth coordination site will determine what color will be produced by the myoglobin molecule (*2*). Deoxymyoglobin, which is in the ferrous (Fe^{2+}) state, is unbound at the sixth coordination site and produces a purple color. Oxymyoglobin is also in the Fe^{2+} state but has oxygen bound to the sixth coordination site. Oxymyoglobin produces the characteristic red color associated with fresh meat. Metmyoglobin results from the oxidation of the iron atom in the heme group, from the Fe^{2+} state to Fe^{3+} state, and produces a brown color that is associated with a loss of freshness. A true brown color will develop in meat when 60% of the myoglobin is in the metmyoglobin state (*1*). Meat that shows signs of browning must be either marked down for quick sale or ground and marketed as a lower value product (*1*). It has been estimated that as much as a billion dollars per year is lost at the retail level due to the browning of fresh meat.

The shelf life of raw meat is determined by both bacterial spoilage and the chemical oxidation of oxymyoglobin (*1*, *3*). In most cases the oxidation of oxymyoglobin to metmyoglobin and subsequent development of brown color usually precedes microbial spoilage (*1*). Many factors are responsible for the oxidation of oxymyoglobin including pH, light exposure, storage temperature, bacterial growth, relative humidity, and lipid oxidation (*3*).

Traditionally there has been much interest in identifying molecules that act as prooxidants in muscle foods. One such molecule, peroxynitrite, is formed by the reaction of nitric oxide, an intracellular messenger, with superoxide, a byproduct of cellular respiration. Peroxynitrite is a relatively stable anion at alkaline pH but at physiological pH has a half-life of ~1 s. The decomposition of peroxynitrite leads to the formation of radical species that can oxidize lipids, proteins, and DNA (*4*). Previous research has shown that peroxynitrite can induce lipid oxidation in skeletal muscle microsomes and homogenates under the conditions expected in muscle foods (*5*). Because nitric oxide synthase can produce nitric oxide in skeletal muscle (*6*), it is possible that peroxynitrite could influence the quality of muscle foods.

The purpose of this research was to study the effect of peroxynitrite on the stability of oxymyoglobin. This study characterizes the effect of concentration, varying pH, temperature, and CO_2 on the ability of peroxynitrite to oxidize oxymyoglobin to metmyoglobin. Differential scanning calorimetry (DSC) analysis was used to evaluate if peroxynitrite affected myoglobin structure.

MATERIALS AND METHODS

Materials. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Peroxynitrite was synthesized by reaction of isoamyl nitrite with 9.0 M H_2O_2 at pH 13 (*7*). Excess H_2O_2 was removed by passage through a column of manganese dioxide. Peroxynitrite was stored at −80 °C and was spectrophotometrically standardized (ϵ = 1670 M^{-1} cm^{-1}) immediately prior to each experiment.

Oxymyoglobin was prepared by reduction of 10 mM metmyoglobin (horse skeletal muscle) in 200 mM phosphate buffer (pH 7.0) with sodium hydrosulfite (0.1 mg of sodium hydrosulfite to 1 mg of myoglobin; *8*). Excess sodium hydrosulfite was removed by passage

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through a Bio-Rad Econopak 10 DG disposable desalting column (Bio-Rad Laboratories, Hercules, CA; exclusion limit = 6000 Da) as follows: Myoglobin solution (0.4 mL) was allowed to pass into the column, and then 3.0 mL of phosphate buffer (200 mM, pH 7.0) was added to elute the myoglobin to the end of the column. Desalted myoglobin was then collected by the addition of 1.5 mL of 200 mM phosphate buffer (pH 7.0). Solutions of oxymyoglobin were made fresh prior to each experiment and kept on ice until use.

Methods. *Reaction of Peroxynitrite with Oxymyoglobin.* Peroxynitrite−oxymyoglobin reactions were carried out in 16 × 100 mm test tubes using a total volume of 1 mL. Each tube contained 50 μL of peroxynitrite (diluted in 0.1 M NaOH to give a final concentration of 25−100 μM peroxynitrite) to which 0.95 mL of oxymyoglobin (final concentration = 0.15 mM) in 200 mM phosphate buffer was added with mixing. The pH (5.5−7.0) of the phosphate buffer was adjusted with NaOH so that the desired pH would be obtained after the addition of the peroxynitrite. Carbon dioxide (1 mM) was incorporated into the system by adding sodium bicarbonate (25 mM) to the oxymyoglobin before the addition of peroxynitrite (*9*). Samples were incubated at 4−35 °C, and the state of myoglobin was determined spectrophotometrically at 450−600 nm using an Ultrospec 3000 Pro spectrophotometer (Biochrom Ltd., Cambridge, U.K.). Metmyoglobin concentrations were calculated according to the method of Krzywicki (*10*).

Differential Scanning Calorimetry. DSC analysis was performed using a VP-DSC (Microcal, Northampton, MA). A 10 mM solution of metmyoglobin was prepared in 200 mM phosphate buffer and then passed through a desalting column as described above for oxymyoglobin. The desalted metmyoglobin was then diluted to 0.15 mM metmyoglobin in 200 mM phosphate buffer. Oxymyoglobin was prepared by reducing a 10 mM solution of metmyoglobin with hydrosulfite and passing through a desalting column as described under Materials. The resulting oxymyoglobin (1.7 mM) was then oxidized with 0.4 mM peroxynitrite to ensure complete conversion to metmyoglobin. Absorbance of this solution, using the methodology described above, was measured to verify that oxymyoglobin was completely converted to metmyoglobin. The oxidized metmyoglobin was then passed through a desalting column as described above to remove residual reactants and was diluted to 0.150 mM using 200 mM phosphate buffer (pH 7.0). Desalted metmyoglobin or peroxynitrite-modified metmyoglobin (0.15 mM) was placed in the sample cell with 200 mM phosphate buffer in the reference cell. Temperature was increased from 10 to 90 °C at a rate of 90 °C/h (*11*).

Reduction of Peroxynitrite-Modified Metmyoglobin. A 1.7 mM solution of oxymyoglobin (produced as described under Differential Scanning Calorimetry) was reacted with 1.2 mM peroxynitrite for 1 h. The resulting metmyoglobin (metMb) was then desalted as above, and 1.0 mM metMb was reduced with 20 mM sodium hydrosulfite with vortexing until the color turned red (~20 s). The resulting oxymyoglobin was then desalted, and metMb formation was measured spectrophotometrically at 25 °C as a function of time. The rate of metMb formation was calculated from the slopes of the lines.

Statistics. All measurements were made on triplicate samples and reported as means ± standard deviations (SD). The general linear model procedure (*12*) was used to test for significance ($p \leq 0.05$). Duncan's multiple-range test was used to separate means (*12*).

RESULTS AND DISCUSSION

Several other studies have been conducted to evaluate the effects of peroxynitrite and related compounds on the conversion of oxymyoglobin to metmyoglobin (*13*−*16*). However, these papers describe experiments in which peroxynitrite concentrations were in great excess to myoglobin concentrations, a situation not likely to occur in muscle foods. Therefore, the purpose of this research was to evaluate the ability of peroxynitrite to promote the conversion of in situ concentrations of oxymyoglobin in beef (0.15 mM) to metmyoglobin under conditions expected in muscle foods.

Reagent peroxynitrite (25−100 μM) caused rapid and extensive formation of metmyoglobin with the majority of metmyoglobin formation occurring during the first 20 min of incubation and a slower rate occurring afterward (**Figure 1**). The amount of metmyoglobin formed increased with increasing peroxynitrite concentrations with 0, 25, 50, 75, and 100 μM peroxynitrite promoting 12, 30, 70, 90, and 95% metmyoglobin formation, respectively, after 90 min of incubation. The concentrations of peroxynitrite needed to promote substantial oxidation of oxymyoglobin (25−100 μM) were much lower than those needed to cause lipid oxidation (0.5−2.0 mM; *5*), suggesting that myoglobin may be a more likely target for the quality deterioration of muscle foods by peroxynitrite.

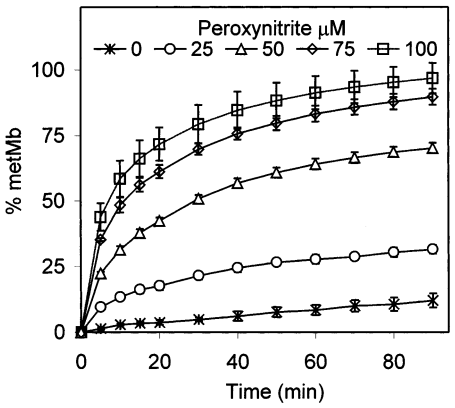

Figure 1. Ability of increasing concentrations of peroxynitrite (0–100 μM) to promote the formation of metmyoglobin (metMb) from oxymyoglobin (0.15 mM) at pH 7.0 and 25 °C. Data points represent means ($n = 3$) ± SD (error bars). Some error bars lie within the data points.

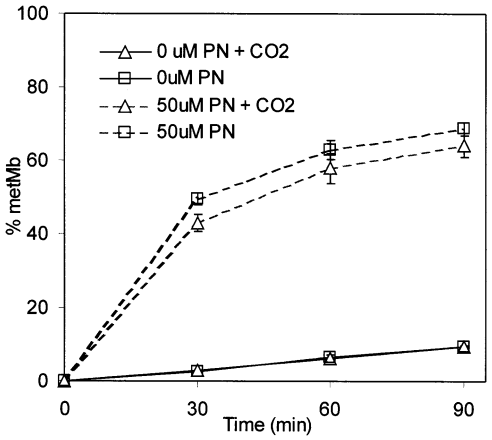

Figure 2. Influence of 1.0 mM carbon dioxide (CO2) on the ability of peroxynitrite (PN, 50 μM) to promote the formation of metmyoglobin (metMb) from oxymyoglobin (0.15 mM) at pH 7.0 and 25 °C. Data points represent means ($n = 3$) ± SD (error bars). Some error bars lie within the data points.

Carbon dioxide can interact with peroxynitrite to form oxidizing species such as the carbonate ($CO_3^{\bullet-}$) and nitrogen dioxide ($NO_2^{\bullet}$) radicals (*9*). These radicals can change the prooxidant potential of peroxynitrite. For instance, carbon dioxide can substantially decrease the oxidation of liposomes and skeletal muscle microsomes by reagent peroxynitrite (*5*). Carbon dioxide by itself had no influence on the conversion of oxymyoglobin to metmyoglobin in the absence of peroxynitrite (**Figure 2**). Addition of the combination of 1 mM carbon dioxide and 50 μM reagent peroxynitrite decreased oxymyoglobin oxidation after 30 min of incubation, with metmyoglobin concentrations being 4−10% less than oxidation by peroxynitrite alone at pH 7.0. Exner and Herold (*14*) found that 2.5 mM carbon dioxide increased peroxynitrite-induced conversion of

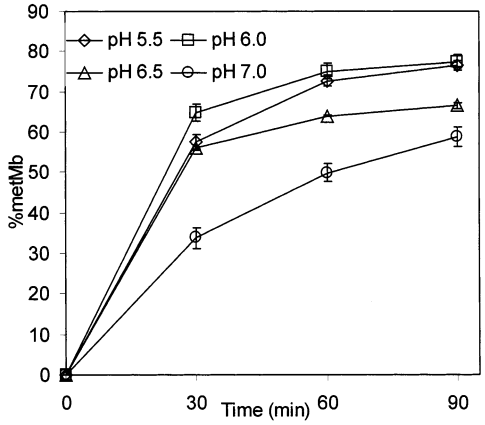

Figure 3. Influence of pH (5.5–7.0) on the ability of peroxynitrite (50 μM) to promote the formation of metmyoglobin (metMb) from oxymyoglobin (0.15 mM) at 25 °C. Data points represent means ($n = 3$) ± SD (error bars). Some error bars lie within the data points.

oxymyoglobin to metmyoglobin at pH 7.3 when peroxynitrite concentrations were in 10-fold excess to myoglobin concentrations.

The prooxidant activity of peroxynitrite can be dramatically influenced by pH because peroxynitrite decomposes rapidly at pH values below its pK_a (6.8; *17*). At the pH values expected in different muscle foods (5.5−7.0), oxymyoglobin exhibits different degrees of stability, with conversion to metmyoglobin increasing with decreasing pH in the absence of peroxynitrite (data not shown; *3*). The effect of pH on peroxynitrite-induced oxymyoglobin oxidation was calculated by subtraction of metmyoglobin values produced in the absence of peroxynitrite from metmyoglobin values in the presence of peroxynitrite. **Figure 3** shows that peroxynitrite-promoted conversion of oxymyoglobin to metmyoglobin increased from pH 7.0 to 6.0 with no additional differences being observed between pH 6.0 and 5.5 ($p > 0.05$). This is in contrast to reagent peroxynitrite, the stability of which decreases with decreasing pH (*17*), and the interaction of peroxynitrite with lipids, where pH (5.6−7.2) change did not dramatically influence lipid oxidation rates (*5*). Exner and Herold (*14*) also found an increase in peroxynitrite-induced oxymyoglobin oxidation rates with decreasing pH (6.4−7.3). Herold et al. (*15*) reported that a peroxynitrite−myoglobin (Fe^{2+}) complex rapidly converts to metmyoglobin at pH < 7.0. This proposed mechanism could also be involved in the increase in peroxynitrite-induced metmyoglobin formation with decreasing pH observed in our system. The effect of temperature on peroxynitrite metmyoglobin formation was also tested because muscle foods are subjected to a wide variety of temperatures. As expected, increasing incubation temperatures increased the extent that peroxynitrite converted oxymyoglobin to metmyoglobin after 30 min of incubation (**Figure 4**).

Peroxynitrite could cause the conversion of oxymyoglobin to metmyoglobin by direct oxidation of the ferrous iron in oxymyoglobin or by modification of protein structure that would result in decreased stability of oxymyoglobin. DSC was used to evaluate whether peroxynitrite was able to alter the protein structure of myoglobin (**Figure 5**). The maximum enthalpy change for metMb was 80.5 °C, whereas oxymyoglobin that had been converted to 100% metmyoglobin by peroxynitrite had a maximum enthalpy change at 80.0 °C. Total heat capacity for the peroxynitrite-modified metMb was similar to that of the metMb control. These results suggest that peroxynitrite did not cause major structural changes in myoglobin.

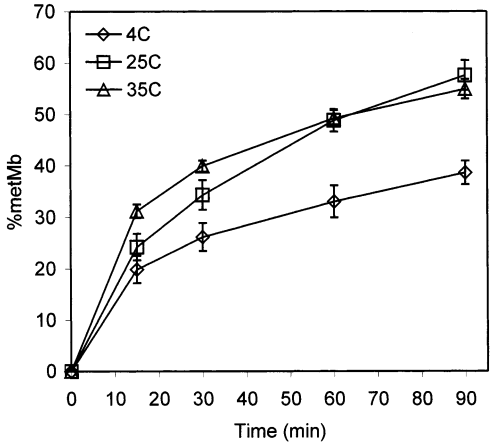

Figure 4. Influence of temperature on the ability of peroxynitrite (50 μM) to promote the formation of metmyoglobin (metMb) from oxymyoglobin (0.15 mM) at pH 7.0. Data points represent means ($n = 3$) ± SD (error bars). Some error bars lie within the data points.

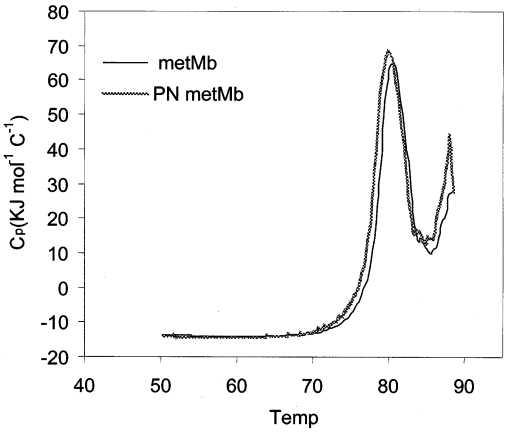

Figure 5. DSC curves of metmyoglobin and peroxynitrite (PN)-modified metmyoglobin.

The stability of peroxynitrite-modified oxymyoglobin was tested by converting oxymyoglobin to 100% metmyoglobin with peroxynitrite. After desalting to remove residual peroxynitrite, the metmyoglobin was converted back to oxymyoglobin with 20 mM hydrosulfite in the presence of air. The stability of this peroxynitrite-modified oxymyoglobin was then compared to that of unmodified oxymyoglobin by measuring metMb formation as a function of time. Addition of 20 mM hydrosulfite to unmodified metmyoglobin resulted in 100% conversion to oxymyoglobin (**Figure 6**). The same concentration of hydrosulfite was only able to convert 90% of the peroxynitrite-modified myoglobin to oxymyoglobin. This observation and the fact that further increasing hydrosulfite concentrations to100 mM did not result in 100% conversion to oxymyoglobin (data not shown) suggest that peroxynitrite was modifying some of the myoglobin in a manner that prevented myoglobin reduction. In a comparison of the stability of the unmodified and peroxynitrite-modified oxymyoglobin, no difference in metmyoglobin formation rates was observed (**Figure 6**). Because the peroxynitrite-modified oxymyoglobin had color stability similar to that of unmodified myoglobin, this again suggests that peroxynitrite was not causing major changes in the conformation of myoglobin (structurally modified oxymyoglobin often has lower color stability than native myoglobin; *18*). If peroxynitrite does not structurally modify myoglobin, this

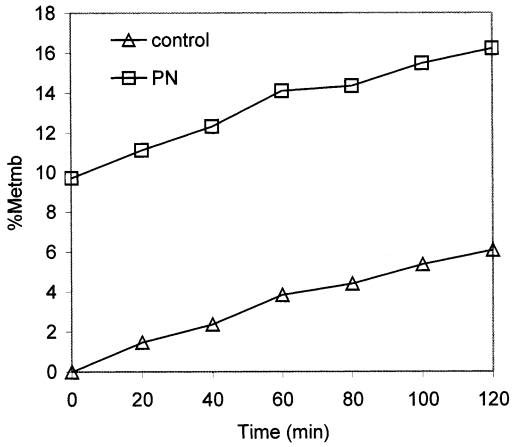

Figure 6. Metmyoglobin formation rates of oxymyoglobin and peroxynitrite (PN)-modified oxymyoglobin (0.15 mM) at pH 7.0 and 25 °C.

suggests that peroxynitrite (or a peroxynitrite breakdown product)-promoted metMb formation was primarily due to direct conversion of Fe^{2+} to Fe^{3+}. This hypothesis is in agreement with Herold et al. (*15*), who reported that peroxynitrite can coordinate with the heme portion of myoglobin to promote metmyoglobin formation without causing nitration of the amino acids in the protein.

CONCLUSIONS

Peroxynitrite is a potential oxidizing agent that could be active in muscle foods. Peroxynitrite is capable of causing the conversion of oxymyoglobin to metmyoglobin at concentrations lower than is required for peroxynitrite-induced oxidation of lipids. Peroxynitrite's ability to promote discoloration of oxymyoglobin increases with decreasing pH and increasing temperatures. The mechanism of peroxynitrite-induced discoloration of oxymyoglobin seems to be through the direct conversion of Fe^{2+} to Fe^{3+} without major structural modification in myoglobin. Overall, these data indicate that peroxynitrite can promote both minor structural alterations and oxidation of oxymyoglobin under the conditions expected in muscle foods, suggesting that peroxynitrite could affect meat color. The results of this study indicate that further research using peroxynitrite specific biomarkers (e.g., nitrotyrosine) is warranted to determine if myoglobin is modified by peroxynitrite in post-mortem skeletal muscle.

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