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Oxidation of Porcine Myosin by Hypervalent Myoglobin: The Role of Thiol Groups

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Oxidation of the myofibrillar muscle protein myosin from pork by hypervalent myoglobin species (MbFe(III)/H_2O_2 radical generating system) was investigated in aqueous solution in the pH range of 5.0–7.8 by electron spin resonance (ESR) spectroscopy using *N*-*tert*-butyl-α-phenylnitrone (PBN) as spin trap and indirectly by determination of the rate of reduction of hypervalent myoglobin species by UV spectroscopy. Cross-linking of myosin was examined by SDS-PAGE. The target for oxidative modification of myosin was studied by thiol blocking by *N*-acetylmaleimide (NEM) and by determining oxidative modification of myosin thiols. The reaction between myosin and hypervalent myoglobin was fast and showed little dependence on pH. The myosin radicals formed were observed to be short-lived. Myosin thiols are suggested to be the main target for oxidative modification, as NEM-treated myosin did not form radicals in the presence of hypervalent myoglobin. A significant decrease in thiol content was already demonstrated 25 s after initiation of oxidation of myosin. The majority of myosin heavy chain (MHC) was demonstrated to be cross-linked through intermolecular disulfide bonding 1 h after initiation of oxidation. This demonstrates that thiols are important for radical formation and cross-linking of myosin during oxidation with hypervalent myoglobin at the pH of meat products.

KEYWORDS: Myosin; hypervalent myoglobin; protein oxidation; radical formation; thiol groups; cross-linking; pH

INTRODUCTION

Oxidative reactions are together with microbial growth important for the quality and shelf life of meat. Reactive oxygen species (such as H_2O_2, $O_2^{\bullet-}$) accumulate during the conversion of muscle to meat, and pro-oxidative species such as hypervalent myoglobin are formed in parallel with depletion of the antioxidative defense of the muscle cells (*1–3*). The meat pigments, myoglobin [MbFe(II)] and oxymyoglobin [MbFe(II)O_2], will oxidize to metmyoglobin [MbFe(III)] when NADH is depleted in the meat. The highly reactive hypervalent myoglobin species, ferrylmyoglobin [MbFe(IV)═O] and perferrylmyoglobin [$^{\bullet}$MbFe(IV)═O], are formed by reaction between MbFe(III) and H_2O_2 (*4–6*). The formation of hypervalent myoglobin in meat has been linked to increased levels of lipid oxidation (*7–9*).

Most studies on oxidation in meat and other food systems have focused on the effects of lipid oxidation (*10–12*). However, proteins, which, besides water, are the major constituents of meat, are also susceptible to oxidative modifications (*13*). A major consequence of protein oxidation is the formation of intermolecular protein cross-links. This includes disulfide bonds formed by oxidation of cysteine thiol groups, dityrosine formation, and reactions between protein carbonyls (formed through oxidation of protein side chains) and the ϵ-amino group of lysine side chains (*13–15*). Hypervalent myoglobin has been shown to be able to induce intermolecular protein cross-linking (*16–20*). Oxidation of proteins has been widely studied in the field of pathology and in relation to human diseases, but the importance of protein oxidation in food systems has so far been less examined.

The oxidation of proteins in meat has previously been suggested to reduce the eating quality of meat by decreased tenderness and juiciness (*21–23*). Recently, intermolecular disulfide cross-linking of myosin was found to take place in pork chops packed in a high-oxygen atmosphere (70% O_2/30% CO_2), and the disulfide cross-linking of myosin was suggested to be responsible for reduced meat tenderization of the pork chops as evaluated by a sensory panel (*24*). In model systems, alterations in myosin conformation and the formation of myosin aggregates through intermolecular cross-linking as a result of oxidation decrease gel-forming ability, protein solubility, and water-holding capacity, which negatively influence sensory characteristics and palatability (*24–27*). Biochemical properties such as the ATPase activity of myosin, which plays a role in muscle contraction, have also been found to change with oxidation of myofibrillar proteins (*28*). The intermolecular cross-linking of meat proteins has mainly been studied in model systems and based on oxidative modification of myofibrillar

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protein fractions by a variety of radical-generating systems but without considering reaction mechanisms and pH dependency (*18, 27, 29, 30*).

We have undertaken a mechanistic study of the oxidation of purified myosin at various pH values in an aqueous model system. Special emphasis has been put on the role of sulfur-centered myosin oxidation by using electron spin resonance (ESR) spectroscopy for the detection of radicals and traditional assays for the evaluation of thiol oxidation. The protein was oxidized with hypervalent myoglobin species, because these oxidants inherently are found in meat following reaction between the meat pigment myoglobin and H_2O_2 that accumulate in meat during storage (*4–6*).

MATERIALS AND METHODS

Chemicals. Adenosine triphosphate (ATP), Coomassie Brilliant Blue G, ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), *N*-ethylmaleimide (NEM), 90% horse heart metmyoglobin type III, 2-[*N*-morpholino]ethanesulfonic acid (MES), and 98% *N*-*tert*-butyl-α-phenylnitrone (PBN) were purchased from Sigma Chemical Co. (St. Louis, MO). Tris(hydroxymethyl)aminomethane (Tris) and L-cysteine were purchased from Merck (Darmstadt, Germany). Standardized 0.2000 M aqueous HCl and 99% sodium dodecyl sulfate (SDS) were purchased from Bie & Berntsen (Rødovre, Denmark). 2,2′-Dithiobis(5-nitropyridine) (DTNP) (96%) was purchased from Aldrich (St. Louis, MO). 2,2,6,6 -Tetramethylpiperidine 1-oxyl (TEMPO) was purchased from Molecular Probes (Leiden, The Netherlands). Hydrogen peroxide (30%) was purchased from Sigma-Aldrich (Steinheim, Germany). Ethylenbis(oxyethylenenitrilo)tetraacetic acid (EGTA) was purchased from ACROS Organics (Fair Lawn, NJ). *N*-[Tris(hydroxymethyl]-methyl)glycine (tricine) was purchased from AppliChem (Darmstadt, Germany). Pure ethanol (96%) was purchased from Danish Distillers (Aalborg, Denmark). Precision Plus Protein Standard was purchased from Bio-Rad Laboratories (Hercules, CA). All chemicals were of analytical grade or of the highest purity available. Water was purified through a Millipore Q-Plus purification train (Millipore Corp., Bedford, MA).

Purification of Myosin. Myosin was isolated as described previously by Nauss et al. (*31*) and Wang and Smith (*32*) with minor modifications. Porcine longissimus dorsi, supplied by The Danish Meat Research Institute, Roskilde, Denmark, was frozen in liquid nitrogen immediately after slaughter and stored at −18 °C. Fifty grams of muscle, trimmed of adhering fat and connective tissue, was homogenized using an Ultra-Turrax and 3 volumes (v/w) of a modified Guba−Straub solution (0.30 M KCl, 0.10 M KH_2PO_4, 0.05 M K_2HPO_4, and 1.0 mM EGTA, pH 6.4). The crude myofibrillar extract was diluted with 3 volumes of water (v/v) and filtered through a 400 μM plastic mesh. Water [6.5 volumes (v/v)] was added to the filtrate, and the samples were allowed to precipitate on ice for at least 2 h. The clear supernatant was decanted, and the remaining precipitate was centrifuged at 2000*g* for 45 min (HiCen21 centrifuge, Herolab, Wiesloch, Germany). The precipitate was subsequently dissolved in 100 mL of 0.50 M KCl, 1.0 mM EGTA, and 10 mM Tris (pH 7.5), treated with 2.0 mM ATP and 5.0 mM $MgCl_2$, and centrifuged at 70000*g* for 30 min (Beckmann Optima TM LE-80IC Ultracentrifuge, Beckman Coulter, Inc., Fullerton, CA) to remove residual actomyosin. Myosin present in the supernatant was salted out by ammonium sulfate precipitation. The fraction precipitating between 38 and 50% saturation was collected and resuspended in a minimal volume of 0.50 M KCl, 1.0 mM EGTA, and 10 mM Tris (pH 7.5) and dialyzed overnight against three changes of the same buffer, without EGTA, using a 6000–8000 g/mol cutoff dialysis tube (Spectra/Por membrane, Spectrum, Rancho Dominguez, CA).

The purity of the obtained myosin solution was evaluated by reducing SDS-PAGE using 3–8% Tris−acetate gels (NuPAGE, Invitrogen, Carlsbad, CA). DTT was added directly to the samples to obtain reducing conditions. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue, allowing visualization of the separated protein bands and comparison to a protein standard (Precision Plus Protein standard, Bio-Rad Laboratories). The myosin solution contained primarily myosin heavy chain, but also small amounts of low molecular weight compounds; for example, the presence of a protein band between 37 and 50 kDa assigned as actin was observed.

The purified myosin stock solution was stored at −18 °C. On the day of use, stock myosin was thawed and kept on ice until application. The myosin concentration was determined by measuring absorbance at 280 nm by use of $A_{280(1g/L)} = 0.496$ (*33*).

Purification of MbFe(III) and Preparation of H_2O_2. MbFe(III) was dissolved in a minimum volume of buffer, filtered by use of a 0.45 μM filter (Minisart, Satorius AG, Uppsala, Sweden), and purified on a PD-10 Sephadex G-25 column (Amersham Biosciences, Uppsala, Sweden). A concentration of approximately 20 mM of the eluted MbFe(III) was determined spectrophotometrically at 525 nm, $\epsilon = 7700$ M^{-1} cm^{-1} (*34*), against a blank consisting of the same buffer. MbFe(III) solutions were stored at 2–5 °C in the dark and used within 3 days. H_2O_2 was diluted in buffer, and concentration was determined spectrophotometrically at 240 nm, $\epsilon = 39.4$ M^{-1} cm^{-1} (*35*), against a blank consisting of the same buffer. A concentration of approximately 20 mM H_2O_2 was prepared, stored at 2–5 °C, and used within a week. The choice of buffer for MbFe(III) purification and H_2O_2 preparation was based on compatibility with specifications of analysis (pH and ionic strength). An Me6.0234.100 combination glass electrode connected to a Methrom 713 pH-meter was used for pH measurements (Methrom, Herisau, Swizerland), and a HP8453 UV–vis diode array spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) was applied in the spectrophotometric analysis.

Determination of Myosin Cross-Linking by SDS-PAGE. Oxidized and nonoxidized myosin samples were analyzed by gel electrophoresis (SDS-PAGE) using NuPAGE Novex 3–8% Tris−acetate gels (Invitrogen, Carlsbad, CA). Oxidized myosin samples were prepared by mixing 13 μM myosin with 75 μM MbFe(III) and 75 μM H_2O_2 (pH 5.0, 6.4, and 7.8, $I = 0.50$, 5% SDS) in this order and incubating for 1 h at 25 °C. MES buffer (20 mM, pH 5.0, $I = 0.50$, 5% SDS) and phosphate buffer (20 mM, pH 6.4 and pH 7.8, $I = 0.50$, 5% SDS) were used as solvents for all reactants to obtain pH 5.0, 6.4, and 7.8 of the oxidized myosin solution. As a control, myosin was incubated for 1 h at the appropriate pH at 25 °C without the addition of MbFe(III) and H_2O_2. Incubations were carried out in Eppendorf tubes, allowing 3 volumes of headspace of air. Samples with 5 μg of protein were loaded directly onto the gel after 1 h of oxidation. DTT was added directly to samples when reducing conditions were employed during electrophoresis. Myosin samples were run in duplicate.

Protein Thiol Groups. Thiol oxidation of myosin was measured spectrophotometrically by a modification of Ellman's method using the thiol-sensitive reagent 2,2′ dithiobis(5-nitropyridine) (DTNP) as modification of the methods described by Winterbourn (*36*) and Morzel et al. (*37*). Free thiol content (nmol/mg myosin) of (i) nonoxidized myosin, (ii) oxidized myosin, and (iii) MbFe(III)/H_2O_2 radical-generating systems was determined at high ionic strength ($I = 0.50$).

Preparation of oxidized and nonoxidized myosin sample was done in accordance with samples prepared for SDS-PAGE analysis. MES buffer (20 mM, 5% SDS, $I = 0.50$) was used for incubation of myosin at pH 5.0 and 5.7, whereas phosphate buffer (20 mM, 5% SDS, $I = 0.50$) was used as solvent for higher pH values (6.4, 7.5, and 7.8). Briefly, thiol determination was carried out by adding 1.00 mL of phosphate buffer (20 mM, 5% SDS, pH 8.0, $I = 0.50$) to the 0.50 mL of thawed oxidized or nonoxidized myosin sample, mixed forcefully (whirled), and heated at 70–80 °C for 10 min to get myosin into solution. Timing was started as soon as the myosin sample was thawed. The heated oxidized myosin sample (120 μL) was mixed with 860 μL of phosphate buffer (20 mM, 5% SDS, pH 8.0, $I = 0.50$). Absorbance was measured at 386 and 700 nm prior to the addition of DTNP in order to subtract the contribution of myosin and myoglobin species to absorbance at 386 nm. Five minutes after heat treatment of the reaction mixture, 20 μL of 5 mM DTNP in absolute ethanol was added, and the absorbance was recorded at 386 and 700 nm 5 min after addition (that is, 20 min after thawing). Phosphate buffer (20 mM, 5% SDS, pH 8.0, $I = 0.50$) was used as blank. pH was measured in the reaction mixture instantly after the addition of DTNP and adjusted with HCl/NaOH if the pH deviated from 8.0 by more than ±0.1. A standard curve was established using 1 mM L-cysteine in 20 mM 5% SDS

phosphate buffer (pH 8.0, $I = 0.50$) in the concentration range of 0–90 μM, with a reference solution consisting of the same buffer (*38*). All samples were measured in triplicate. Thiol content was calculated as nanomoles of cysteine per milligram of myosin.

Detection of Myosin Oxidation by Spin Trapping. Myosin oxidation was carried out at room temperature (pH 7.0, $I = 0.16$). Equimolar amounts of MbFe(III) and H_2O_2 were mixed prior to the addition of myosin and PBN, reaching final concentrations of 300 μM MbFe(III), 300 μM H_2O_2, 0, 11.8, 23.5, and 46.8 μM myosin and 30 mM PBN. The order of mixing the reactants was investigated and was found not to have a significant influence on the obtained ESR spectra. Phosphate buffer (5.0 mM, pH 6.9, $I = 0.16$) was used as solvent for all reactants, and the pure buffer solution was used as blank. The samples were whirled, and the ESR spectra of samples (50 μL) in micropipets (Brand GmbH, Wertheim, Germany) were recorded at room temperature 30 s after mixing of MbFe(III) and H_2O_2. A MiniScope MS 200 ESR spectrometer (Magnettech, Berlin, Germany) was used with the following settings: microwave power, 10 mW; sweep width, 97.76 G; sweep time, 120 s; modulation amplitude, 2 G; and time constant, 0 s. An aqueous solution of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) (2 μM) was used as a reference sample to calculate the absolute radical concentration measured in the sample by double integration of the ESR spectra. The TEMPO standard was measured as the first and last samples of the day, and all samples were measured in triplicate. Bruker Win-EPR software (version 2.11, Bruker-Franzen, Analytik GmbH, Germany) was applied for data treatment of ESR spectra.

Ionic strength (0.16, 0.25, and 0.50, respectively) and pH effects ($5.0 < \text{pH} < 7.8$) on myosin oxidation were determined at room temperature. The ionic strength of the myosin stock solution were obtained by dialysis in a 100 times volume of 20 mM MES buffer (pH 5.0) or 20 mM phosphate buffer (pH 6.5 and 7.5) depending on the choice of ionic strength and the pH of the myosin solution and using a dialysis tube with a 6000–8000 g/mol cutoff (Spectra/Por membrane, Spectrum) at 2–5 °C during >3 h against three changes of the same buffer. Stock solutions of MbFe(III), H_2O_2, and PBN were prepared using a buffer matching the desired pH and ionic strength of investigation.

Blocking of Free Thiols by NEM. Chemical modification of thiol groups in myosin was carried out by use of NEM, according to a modification of the methods by Smyth et al. (*39, 40*) and Wada and Kitabatake (*41*). Accessible thiol groups of 23.5 μM myosin were blocked by incubation with 0, 1.0, 2.5, and 5.0 mM NEM for 90 min on ice and protected from light. Subsequently, oxidation of NEM-treated and untreated myosin was carried out at room temperature (pH 7.0, $I = 0.50$), and ESR spectra were recorded under the conditions previously mentioned. Myosin sample and PBN were added simultaneously to MbFe(III) and H_2O_2, reaching final concentrations of 300 μM MbFe(III), 300 μM H_2O_2, 30 mM PBN, and 23.5 μM myosin. Phosphate buffer (5.0 mM, pH 7.0, $I = 0.50$) was used as solvent for all reactants. Radical formation by the MbFe(III)/H_2O_2 radical-generating system was performed as a control. A final concentration of 300 μM MbFe(III) was treated with 0, 1.0, 2.5, and 5.0 mM NEM for 90 min kept on ice. Measurements in the presence of 30 mM PBN were started 30 s after activation of NEM-treated MbFe(III) by H_2O_2 in a final concentration of 300 μM H_2O_2. A blank consisting of phosphate buffer (5.0 mM, pH 7.0, $I = 0.50$) was measured. All measurements were done in triplicate.

Reduction of Hypervalent Myoglobin Species by Myosin. Reduction of hypervalent myoglobin species was investigated in the presence and absence of myosin at 25 °C (pH 7.5, $I = 0.16$) by recording spectral changes between 450 and 700 nm for up to 60 min. Upon mixture of equal concentrations of MbFe(III) with H_2O_2, perferrylmyoglobin [$^{\bullet}$MbFe(IV)═O)] is formed together with ferrylmyoglobin [MbFe(IV)═O]. Reduction of both hypervalent myoglobin species by myosin was measured by the addition of myosin 5 s after mixing MbFe(III) and H_2O_2, to yield final concentrations of 100 μM MbFe(III), 100 μM H_2O_2, and 0, 1.9, 3.9, 7.8, and 11.6 μM myosin in the reaction mixture. Reduction of MbFe(IV)═O by myosin in the absence of $^{\bullet}$MbFe(IV)═O was obtained by allowing the reaction mixture to age for 10 min at room temperature prior to the addition of myosin (*42*). Stock solutions of MbFe(III) and H_2O_2 were prepared using 5.0 mM phosphate buffer (pH 7.5, $I = 0.16$) as solvent. The sample was whirled and recorded 100 s after the addition of myosin. Blanks were prepared by substitution of the myosin solution with buffer. A HP8453 UV–vis diode array spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) equipped with a temperature-controlled cuvette compartment adjusted to 25 °C (Hetrofrig thermo, Birkerød, Denmark) was used. The concentration of hypervalent myoglobin [[MbFe(IV)═O] + [$^{\bullet}$MbFe(IV)═O]] was determined according to the following expression (*43*):

$$\text{hypervalent myoglobin} = -62(A_{490} - A_{700}) + 242(A_{560} - A_{700}) - 123(A_{580} - A_{700}) \quad (1)$$

The pH of the sample mixtures was measured after completion of the reaction. All samples were run in duplicate.

Statistical Analysis. Statistical analysis was performed using the SAS 8.2 package, SAS Institute, Inc., Cary NC. Data were analyzed by analysis of variance to determine the significance of main effects (e.g., oxidation time, pH). Significant ($p < 0.05$) differences between means were identified by the least significant difference (LSD) procedure.

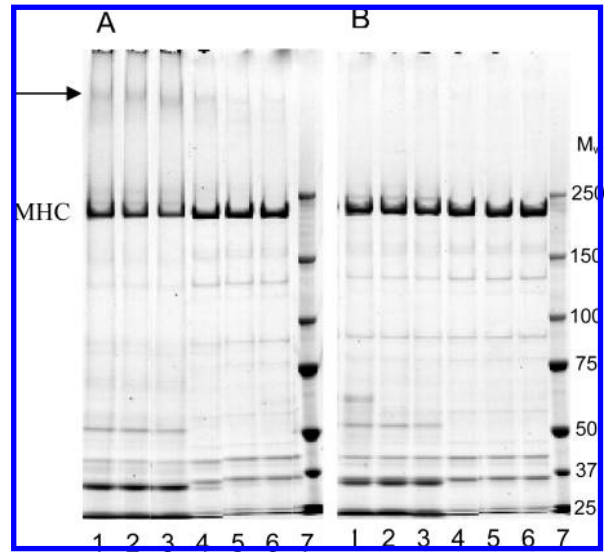

Figure 1. SDS-PAGE of myosin oxidized by hypervalent myoglobin species. Myosin (13 μM) was incubated 1 h at room temperature with MbFe(III) (75 μM) and H_2O_2 (75 μM) at pH 5.0 (lane 1), pH 6.4 (lane 2), and pH 7.8 (lane 3) and without MbFe(III) and H_2O_2 at pH 5.0 (lane 4), pH 6.4 (lane 5), and pH 7.8 (lane 6) at nonreducing conditions (gel **A**) and reducing conditions (gel **B**). Buffers (20 mM, $I = 0.50$) contained 5% SDS during the oxidation. Five micrograms of myosin was loaded in each lane. Lane 7 contained molecular weight standard. MHC, myosin heavy chain.

RESULTS

Intermolecular Cross-Linking of Myosin. Purified myosin was oxidized with hypervalent myoglobin species for 1 h at 25 °C at pH 5.0, 6.4, and 7.8 in the presence of SDS to avoid precipitation of myosin at the lower pH values. The hypervalent myoglobin species were generated in situ by mixing hydrogen peroxide and metmyoglobin. Analysis of the reaction mixtures by SDS-PAGE under nonreducing conditions demonstrated that the intensity of the protein band from myosin heavy chain (MHC) was reduced in comparison to controls consisting of nonoxidized myosin (**Figure 1A**, comparison between lanes 1–3 and 4–6 for oxidized and unoxidized myosin, respectively). Additionally, a protein band appeared at high molecular weight in the oxidized myosin samples (indicated by an arrow in **Figure 1A**), demonstrating that myosin had been cross-linked due to oxidation. Densitometric quantification of the SDS-PAGE bands

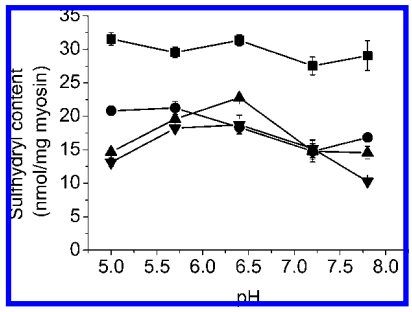

Figure 2. Free thiol groups on myosin after oxidation with hypervalent myoglobin species. Myosin (46.8 μM) was oxidized with 75 μM MbFe(III) and 75 μM H_2O_2 for 25 s (●), 1 h (▲), and 24 h (▼). Control experiments were performed with myosin, but without MbFe(III) and H_2O_2, after 1 h (■). All incubations were carried out at 25 °C (pH 5.0–7.8, $I = 0.50$).

showed that the extents of oxidation of MHC at the three pH values were not significantly different. The cross-linking of myosin was more pronounced after oxidation for 24 h, when a complete loss of MHC has previously been reported (*17*). In the present study myosin was oxidized for a relatively short period of time in order to avoid too extensive losses of MHC that would level any potential effects of pH.

Reducing the samples with DTT during the SDS-PAGE analysis gave only a small reduction in intensity of the MHC protein band of oxidized myosin as compared to nonoxidized myosin (**Figure 1B**, lanes 1–3 and 4–6, respectively). Again, densitometric quantification did not show any significant differences in intensities of the MHC bands from the samples oxidized at the different pH values. The cross-linked MHC band indicated by an arrow in **Figure 1A** was not observed when the SDS-PAGE analysis was made with reducing conditions. The fact that more extensive cross-linking was detected under nonreducing conditions than under reducing conditions suggests that primarily disulfide bonds were responsible for the cross-linking of oxidized MHC. However, cross-linking of MHC also occurred to a small extent via covalent bonds other than disulfide bonding (e.g., dityrosine or Schiff base cross-linking), because a small band of cross-linked MHC was observed under the SDS-PAGE reducing conditions (compare panels **A** and **B** of **Figure 1**). The cross-linking of myosin was clearly not affected by pH as no difference in the MHC band intensities was found in the pH interval 5.0–7.8 at either reducing or nonreducing conditions.

Oxidation of myosin in the absence of SDS resulted in precipitation of myosin at pH 5.0, making a study of the effect of pH in more acidic solutions on myosin cross-linking impossible. Myosin did not precipitate in the absence of SDS during oxidation for pH >6.0, but no difference was observed between pH 6.1 and pH 7.6 on myosin cross-linking in a control experiment performed without the presence of SDS (data not shown).

Effect of Oxidation on Myosin Thiol Content. The role of thiol groups during the oxidation of myosin by hypervalent myoglobin was further investigated by studying the amount of free thiol groups. The determination of myosin thiols was performed with myosin samples oxidized from 25 s to 24 h (pH 5.0–7.8, $I = 0.50$) and in the presence of 5% SDS to avoid myosin precipitation at the lower pH value. The thiol determination showed a significantly higher amount of free thiols in nonoxidized myosin (approximately 30 nmol of thiols/mg of myosin) in comparison to oxidized myosin ($p < 0.001$) (**Figure 2**). The major change in the amount of free thiol groups took place within the first 25 s, and subsequent changes (after 1 and 24 h) were small and observed only at the highest and lowest pH values. Myosin oxidized for 25 s showed a minor pH tendency with a significantly larger decrease in the thiol content during the oxidation at pH 7.2 in comparison to pH 5.7 ($p < 0.05$). The greatest decrease in thiols of approximately 19 nmol of thiols/mg of myosin was observed at pH 7.8 after 24 h of oxidation, equivalent to a 65% decrease in myosin thiols. After 24 h of oxidation, a significantly higher decrease in myosin thiols at pH 5.0 and 7.8 was observed compared to intermediate values ($p < 0.001$). A control experiment with oxidized myosin at pH 6.1 and 7.6 in the absence of SDS confirmed that oxidation of thiols in myosin is a fast reaction with a similar pH dependency as in the presence of SDS (data not shown).

Detection of Radicals by ESR. Radicals were detected during oxidation of myosin by hypervalent myoglobin using the spin trapping technique with detection by ESR spectroscopy (**Figure 3**). The ESR spectra of the PBN spin adducts had features that closely resembled nitroxide centered powder spectrum, which suggests the PBN adducts have a low rotational mobility and therefore most likely are formed by trapping of protein-centered radicals. Due to the lack of resolvable hyperfine coupling constants in the obtained ESR spectra, the exact identity of the initial protein radical cannot be determined. However, in the study by Könczöl et al. (*44*) a similar myosin−PBN spin adduct was formed by oxidation with Ce(IV), which was assigned to a thiyl radical. The concentration of PBN spin adducts increased linearly as a function of myosin concentration ($R^2 = 0.999$) at room temperature (pH 7.4, I = 0.50) (**Figure 3**), which demonstrates that the formation of spin adducts is closely linked to the oxidation of myosin.

The role of myosin thiol groups on the oxidation of myosin was examined by blocking the cysteines in myosin with NEM. A significant decrease ($p < 0.001$) in the amount of PBN spin adducts was observed when NEM-treated myosin was oxidized in comparison to oxidation of untreated myosin (**Figure 4**). At high concentrations of NEM the same concentrations of PBN spin adducts were formed by the hypervalent myoglobin species in the presence and in the absence of myosin ($p > 0.05$). This demonstrates that the thiol groups on myosin play an important role in the formation of radicals during the oxidation of myosin. NEM treatment had only a small effect on the MbFe(III)/H_2O_2 radical-generating system as a minor decrease in radical concentration of NEM-treated spin adducts was observed in comparison to radical concentrations without the addition of NEM. These myoglobin-derived spin adducts are most likely due to reactions with histidine or α-amino groups of MbFe(III) (*39, 40*). $^{\bullet}$MbFe(IV)=O radical species are known to be either tyrosine- or tryptophan-centered (*45*), and the pro-oxidative effects of the MbFe(III)/H_2O_2 radical generating system are therefore not expected to be strongly affected by NEM.

Effect of pH and Ionic Strength on Myosin Radical Formation. The ionic strength (0.16, 0.25, and 0.50) and pH (5.1–7.4) affected the amount of detected spin adducts during the oxidation of myosin oxidation (**Figure 5A**). The highest amounts of myosin−PBN spin adduct were detected at the high ionic strength, at which myosin exists primarily as free monomers, in contrast to lower ionic strength, at which the majority of myosin is bound as dimers and synthetic filaments (*46–48*). The PBN spin adduct concentration of myosin-derived radicals increased linearly with increasing pH at all three ionic strengths. Control studies in which the hypervalent myoglobin

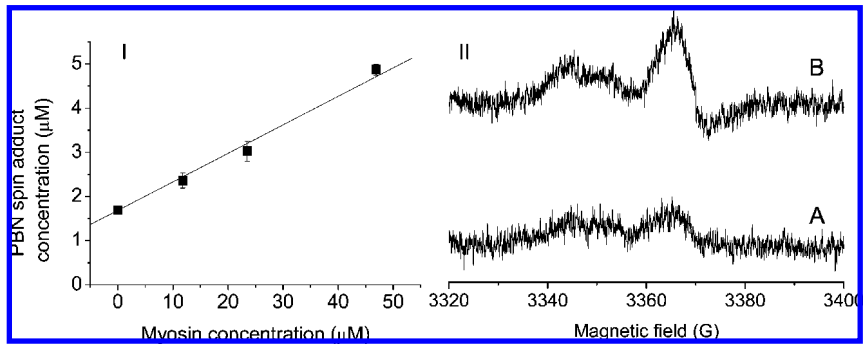

Figure 3. (**I**) Radical formation (μM PBN spin adducts) demonstrating oxidation of myosin by hypervalent myoglobin species. Myosin (0–46.8 μM) was oxidized by 300 μM MbFe(III) and 300 μM H_2O_2 at room temperature (pH 7.4, $I = 0.50$). (**II**) ESR spectra of control with MbFe(III)/H_2O_2/PBN (**A**) and myosin/MbFe(III)/H_2O_2/PBN (**B**) were recorded 25 s after initiation of oxidation at room temperature.

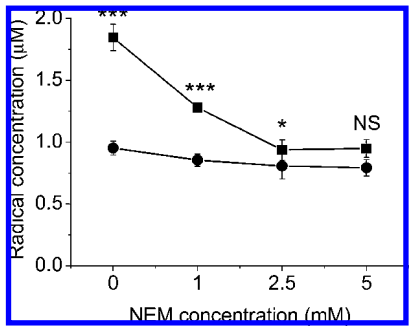

Figure 4. Effect of thiol blocking by NEM on the formation of PBN spin adducts during oxidation of myosin by hypervalent myoglobin species. Myosin (23.5 μM) was treated with NEM (0–5 mM) before mixing with MbFe(III) (300 μM), H_2O_2 (300 μM), and PBN (30 mM) and ESR detection of spin adducts (■). Control experiments were performed without myosin (●). ESR spectra were recorded 25 s after initiation of oxidation at room temperature (pH 7.0, $I = 0.50$) by the presence of 30 mM PBN.

species were generated in the absence of myosin gave significantly lower amounts of spin adducts at all ionic strengths and at all pH values except at pH 5.25 and $I = 0.16$ (**Figure 5B**).

Reduction of Hypervalent Myoglobin Species by Myosin. The hypervalent myoglobin species used in the present study to oxidize myosin are a mixture of $^{\bullet}$MbFe(IV)═O and MbFe(IV)═O together with unreacted MbFe(III) as generated by the activation of MbFe(III) with H_2O_2 (*4, 9, 49*). $^{\bullet}$MbFe(IV)═O and MbFe(IV)═O are both able to oxidize proteins; however, their reactivities and pH dependencies are very different (*42*). Kinetic studies of $^{\bullet}$MbFe(IV)═O reactions are complicated because the formation of $^{\bullet}$MbFe(IV)═O is slow compared to the reactions by which it decays. MbFe(IV)═O is long-lived, and kinetic studies of the reactions of this species are therefore more reliable. The reactions between (i) $^{\bullet}$MbFe(IV)═O/MbFe(IV)═O and myosin and between (ii) MbFe(IV)═O and myosin were studied by UV–vis spectroscopy at 25 °C (pH 7.45, $I = 0.16$). It is well-known that the reactions of MbFe(IV)═O are pH-dependent (*9*). However, determination of the effect of pH on the reduction of MbFe(IV)═O by myosin was not possible as myosin precipitates at lower pH values. The product spectra showed that MbFe(IV)═O was reduced to MbFe(III) both in the absence (autoreduction) and in the presence of myosin (**Figure 6**). Reduction of MbFe(IV)═O was clearly found to be accelerated by the presence of myosin in comparison to the autoreduction of MbFe(IV)═O as evidenced by the faster decrease in absorbance at 525–625 nm and faster increase around 500 and 650 nm in the presence of myosin.

The amount of MbFe(IV)═O reduced after 1 h increased with the concentration of myosin (**Figure 7**). This experiment could be carried out with myosin concentrations only up to 12 μM due to the limited solubility of myosin. Hence, an excess of hypervalent myoglobin species relative to myosin had to be used for these studies. A similar experiment was carried out by having myosin present during the activation of metmyoglobin with H_2O_2. However, in this experiment the concentration of hypervalent myoglobin species after 1 h was nearly independent of the myosin concentration. As $^{\bullet}$MbFe(IV)═O has the same spectral characteristics as MbFe(IV)═O it is not possible to separate the two species by UV–vis spectroscopy. In the presence of both hypervalent myoglobin species, myosin reacted primarily with $^{\bullet}$MbFe(IV)═O to form MbFe(IV)═O, as no change in the total concentration of hypervalent myoglobin species was observed. On the other hand, in the experiment containing only MbFe(IV)═O, a decrease in the concentration of hypervalent myoglobin species was observed, which is caused

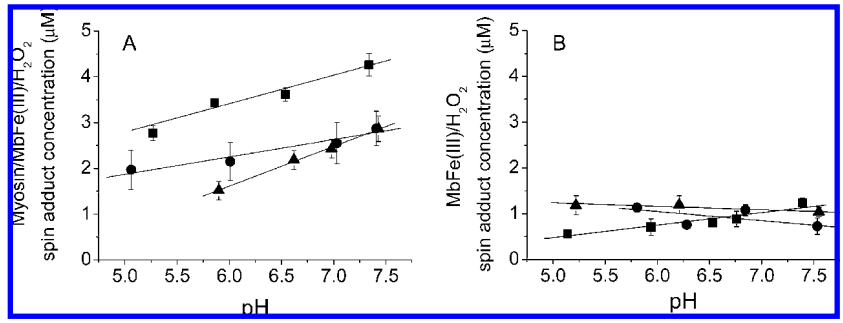

Figure 5. Effects of pH and ionic strength on the formation of spin adducts during myosin oxidation by hypervalent myoglobin species. Spin adducts were detected by ESR 25 s after mixing myosin (46.8 μM) with MbFe(III) (300 μM), H_2O_2 (300 μM), and PBN (30 mM) in buffers with ionic strengths $I = 0.50$ (■), 0.25 (●), and 0.16 (▲) (**A**). Control experiments were performed without myosin (**B**).

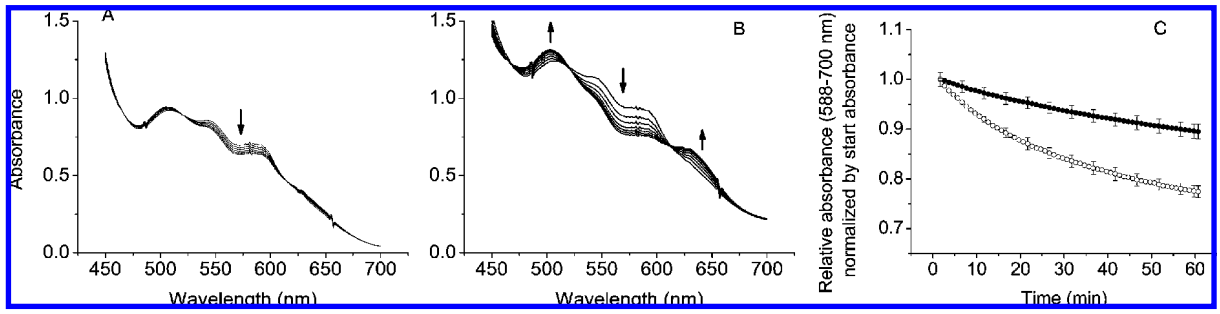

Figure 6. Spectral change (450–700 nm) of MbFe(IV)=O in (**A**) the absence of myosin (autoreduction) and (**B**) the presence of 11.2 μM myosin. The reaction was followed at 25 °C (pH 7.5, $I = 0.16$) for 60 min. MbFe(IV)=O was prepared by mixing equal concentrations of MbFe(III) and H_2O_2 corresponding to final concentrations of 100 μM. The direction of spectral change in relation to the initial spectrum is indicated by an arrow. (**C**) Relative absorbance (588–700 nm) of MbFe(IV)=O as a function of time (minutes) in the absence of myosin (●) and in the presence of 11.2 μM myosin (○).

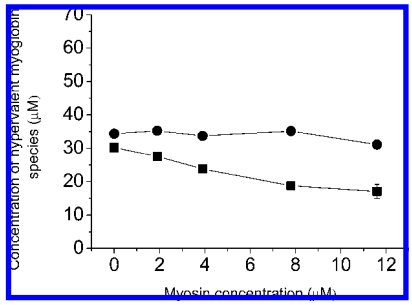

Figure 7. Concentration of hypervalent myoglobin species after mixing MbFe(IV)=O (30 μM) with myosin (■) and after mixing MbFe(III) (100 μM) with H_2O_2 (100 μM) in the presence of myosin (●). Concentrations are calculated on the basis of eq 1 after 60 min of reaction in the presence of 0–12 μM myosin at 25 °C (pH 7.5, $I = 0.16$).

by the reduction of MbFe(IV)=O to MbFe(III) in the presence of a sufficient concentration of myosin when no $^{\bullet}$MbFe(IV)=O is present.

DISCUSSION

Thiols are sensitive to oxidation and are generally believed to be a major target for oxidation in proteins (*13*). The present study has shown that thiol groups play an important role when myosin is oxidized by hypervalent myoglobin species. The oxidation has been shown to lead to the formation of disulfide cross-linked polymers of myosin and a corresponding major reduction of the amount of free thiol groups. The amount of radicals detected by the spin trapping technique during the oxidation of myosin was proportional to the myosin concentration, indicating that the detected spin adducts were myosin-derived radicals. Additionally, blocking of thiol groups with NEM reduced the amount of spin adducts, indicating that the formed myosin radical species were thiol-derived. Thiol-centered radical formation on myosin is consistent with the study of Könczöl et al. (*44*), who demonstrated that myosin thiols are involved in the oxidation of myosin by reaction with the oxidizing agent Ce(IV). The proposed thiol-derived myosin radicals, initially generated by hypervalent myoglobin species, were observed to be short-lived, as evaluated by ESR spectroscopy. Due to the reactivity of the formed myosin radicals and the rapid loss of thiol groups in myosin, as demonstrated in the present study, cross-linking of myosin is likely to occur immediately upon radical formation. It is noteworthy that the ESR study of myosin radical formation and thiol determination, both reflecting early oxidative events, and the study of intermolecular disulfide cross-linking upon myosin oxidation, reflecting late events (end product formation), support the same conclusion of thiols as the reactive site in myosin.

The reaction between $^{\bullet}$MbFe(IV)=O and myosin has previously been found to be concentration-dependent (reaction 2) (*17*). In the present study a radical-generating system consisting of exclusively MbFe(IV)=O was applied, and reduction of MbFe(IV)=O to MbFe(III) was found to depend on myosin concentration, indicating that MbFe(IV)=O reacts directly with myosin (reaction 3). Small amounts of hypervalent myoglobin species, whether $^{\bullet}$MbFe(IV)=O or MbFe(IV)=O, may therefore efficiently be reduced to MbFe(III) if myosin is present in excess concentrations, as is normally the case in meat.

$$\text{myosin-SH} + {}^{\bullet}\text{MbFe(IV)=O} \rightarrow \text{myosin-S}^{\bullet} + \text{MbFe(IV)=O} + \text{H}^{+} \quad (2)$$

$$\text{myosin-SH} + \text{MbFe(IV)=O} + \text{H}^{+} \rightarrow \text{myosin-S}^{\bullet} + \text{MbFe(III)} + \text{H}_2\text{O} \quad (3)$$

The observed intermolecular disulfide cross-link of myosin, formed as the major oxidation product after treatment of myosin with hypervalent myoglobin species, has also been demonstrated in model systems exposing myofibrillar protein fractions or purified myosin to hydroxyl radical-generating systems (*27, 29, 30*). In contrast, hypervalent myoglobin species have previously been reported to mainly cause formation of intermolecular dityrosine bonds in myosin, but notably, formation of disulfide cross-linking was not investigated (*16, 18*). However, in the study by Morzel et al. (*37*), dityrosine formation and loss of thiol groups was observed after oxidation of myofibrillar proteins with a hydroxyl radical-generating system, indicating that both disulfide and dityrosine formation might have occurred. In the present study and in our previous study (*17*) both reducible (disulfides) and nonreducible (assigned to dityrosine) cross-links were observed in oxidized myosin samples, indicating oxidation of other amino acid side chains apart from the cysteine.

During the first 25 s of reaction (referred to as early oxidation events in the following), myosin was concluded to be more sensitive toward oxidation by hypervalent myoglobin species at physiological pH (~7.4) than at pH representative for meat products (pH 5.5–6.0). This conclusion was based on the increased formation of myosin−PBN spin adducts (**Figure 5**) and a significantly higher loss of thiol groups in myosin at pH 7.2 in comparison to 5.7 ($p < 0.05$) 25 s after the initiation of oxidation (**Figure 2**). Increased oxidative modification of myosin at physiological pH is consistent with myosin thiols to be of

importance regarding initiation of oxidation by hypervalent myoglobin species. The thiol anion (RS^-) is the most reactive form of sulfur compounds (*50*), and conversion of myosin thiols into its deprotonated form (RS^-) is more pronounced at physiological pH in comparison to pH representative for meat systems (pK_a of cysteine in proteins is 9–9.5) (*51*). In contrast, MbFe(IV)═O was expected to be a stronger oxidant toward myosin at pH representative of meat systems in comparison to physiological pH [pK_a of MbFe(IV)═O is 5.2 as determined in a study based on 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonate) (*52*)], as the protonated form of MbFe(IV)═O is more reactive toward reducing substrates in comparison to the nonprotonated MbFe(IV)═O (*42, 53, 54*). However, increased oxidative modification of myosin at pH 5.5–6.0 compared to pH 7 was not observed in the present study during the early oxidation events, as evaluated by myosin−PBN spin adduct formation (**Figure 5**) and decrease in thiol concentration (**Figure 2**) 25 s after the initiation of oxidation. The reaction between •MbFe(IV)═O and myosin is fast, and the reactivity of •MbFe(IV)═O toward other proteins has previously been found to be less pH dependent compared to the reactivity of MbFe(IV)═O (*42*). The lack of increased oxidation of myosin at lower pH values observed in the present study may be due to the fact that both hypervalent myoglobin species are present in the reaction solution, with •MbFe(IV)═O being more reactive than MbFe(IV)═O as discussed previously.

As for the late oxidation events, additional loss of thiol groups in myosin was observed after 1 and 24 h at pH 5.0 and 7.8, but not at the intermediate pH values studied ($p < 0.001$). At pH representative of meat systems (5.7), a 28% decrease of myosin thiols was observed 25 s after the initiation of oxidation, in comparison to a 38% decrease obtained after 24 h. This suggests that thiol-centered myosin oxidation occurs shortly after the initiation of oxidation and increases only slightly up to 24 h. This indicates that the early oxidation events are more pH dependent than the late oxidation events for myosin. Consistently, no difference in the extent of intermolecular myosin disulfide bonding was observed 1 h after oxidation of myosin at pH 5.0, 6.4, and 7.8 by SDS-PAGE analysis. Hence, the choice of pH of the model system to simulate conditions of meat was demonstrated to be of minor importance for the oxidative sensitivity of myosin.

The ionic strength of the model system was found to be important for the oxidative sensitivity of myosin. At $I = 0.50$ the majority of myosin is present as monomers (*48*), which were demonstrated to be more sensitive toward oxidation in comparison to the synthetic myosin filaments (mainly present at $I = 0.16$) as higher concentrations of myosin radical spin adducts were detected at $I = 0.50$ than at $I = 0.16$. This difference could be explained by better access of hypervalent myoglobin species to the myosin molecule at higher ionic strength, at which the majority of myosin is present as monomers.

Mainly on the basis of ESR spectroscopy it was possible to identify two steps in myosin oxidation by hypervalent myoglobin species at pH values that are relevant to meat during storage. The initial step including radical formation was somewhat dependent on pH but strongly dependent on ionic strength, whereas the following step involving cross-linking of myosin was independent of pH. Early oxidation events (radical formation and loss of thiols shortly after the initiation of oxidation) regarding myosin were more pH dependent than late oxidation events (cross-linking and loss of thiols ≤1 h after initiation of oxidation). Hence, myosin thiols are suggested to be the main target of oxidative modification by hypervalent myoglobin species, resulting in intermolecular disulfide cross-linking, which has previously been related to reduced tenderness of meat (*24*) and changed functional properties of meat products (*25–27*).

ABBREVIATIONS USED

ATP, adenosine triphosphate; DTNP, 2,2′-dithiobis(5-nitropyridine); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; ESR, electron spin resonance; MbFe(III), metmyoglobin; MbFe(IV)═O, ferrylmyoglobin; •MbFe(IV)═O, perferrylmyoglobin; MES, 2-[*N*-morpholino]ethanesulfonic acid; NEM, *N*-ethylmaleimide; PBN, *N*-*tert*-butyl-α-phenylnitrone; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS−polyacrylamide gel electrophoresis; TEMPO, 2,2,6,6-tetramethylpiperidine 1-oxyl; tricine, *N*-[tris(hydroxymethyl]methyl)glycine; Tris, tris(hydroxymethyl)aminomethane.

LITERATURE CITED

(1) Skibsted, L. H. Chemical changes in meat and meat products during storage, transportation and retail display—theoretical considerations. In *Meat Quality and Meat Packaging*; Taylor, S. S., Raimundo, A., Severini, M., Smulders, F. J. M., Eds.; ECCEAMST: Utrecht, The Netherlands, 1996; pp 169–181.

(2) Mahoney, D. J.; Kaczor, J. J.; Bourgeois, J.; Yasuda, N.; Tarnopolsky, M. A. Oxidative stress and antioxidant enzyme upregulation in SOD1-G93A mouse skeletal muscle. *Muscle Nerve* **2006**, *33*, 809–816.

(3) Harel, S.; Kanner, J. Hydrogen peroxide generation in ground muscle tissue. *J. Agric. Food Chem.* **1985**, *33*, 1186–1188.

(4) King, N. K.; Winfield, M. E. The mechanism of metmyoglobin oxidation. *J. Biol. Chem.* **1963**, *238*, 1520–1528.

(5) Davies, M. J. Identification of a globin-free radical in equine myoglobin treated with peroxides. *Biochim. Biophys. Acta: Bioenerg.* **1991**, *1077*, 86–90.

(6) Egawa, T.; Shimada, H.; Ishimura, Y. Formation of compound I in the reaction of native myoglobin with hydrogen peroxide. *J. Biol. Chem.* **2000**, *275*, 34858–34866.

(7) Baron, C. P.; Andersen, H. J. Myoglobin-induced lipid oxidation. A review. *J. Agric. Food Chem.* **2002**, *50*, 3887–3897.

(8) Carlsen, C. U.; Møller, J. K. S.; Skibsted, L. H. Heme-iron in lipid oxidation. *Coord. Chem. Rev.* **2005**, *249*, 485–498.

(9) Kroger-Ohlsen, M.; Carlsen, C. U.; Andersen, M. L.; Skibsted, L. H. *Pseudoperoxidase Activity of Myoglobin: Pigment Catalyzed Formation of Radicals in Meat Systems*; Morelle, M., Shahidi, F., Ho, C., Eds.; ACS Symposium Series 807; American Chemical Society: Washington, DC, 2002; pp 138–150.

(10) Kanner, J. Oxidative processes in meat and meat-products—quality implications. *Meat Sci.* **1994**, *36*, 169–189.

(11) Skibsted, L. H.; Mikkelsen, A.; Bertelsen, G. Lipid-derived off-flavours in meat. In *Flavour of Meat, Meat Products and Seafoods*, 2nd ed.; Shahidi, F., Ed.; Blackie Academic and Professional: Glasgow, Scotland, 1998; pp 217–256.

(12) Monahan, F. J. Oxidation of lipids in muscle foods: fundamental and applied concerns. In *Antioxidants in Muscle Foods*; Decker, E., Ed.; Wiley: Chichester, U.K., 2003; pp 3–23.

(13) Davies, M. J.; Dean, R. T. *Radical-Mediated Protein Oxidation*; Oxford Science Publications: Oxford, U.K., 2003.

(14) Stadtman, E. R.; Berlett, B. S. Reactive oxygen-mediated protein oxidation in aging and disease. *Chem. Res. Toxicol.* **1997**, *10*, 485–494.

(15) Armstrong, D. A. Thermochemistry of sulfur radicals. In *S-Centered Radicals*, 1st ed.; Alfassi, Z. B., Ed.; Wiley: West Sussex, U.K., 1999; pp 27–62.

(16) Hanan, T.; Shaklai, N. Peroxidative interaction of myoglobin and myosin. *Eur. J. Biochem.* **1995**, *233*, 930–936.

(17) Lund, M. N.; Luxford, C.; Skibsted, L. H.; Davies, M. J. Oxidation of myosin by heme proteins generates myosin radicals and protein cross-links. *Biochem. J.* **2008**, *410*, 565–574.

(18) Hanan, T.; Shaklai, N. The role of H_2O_2-generated myoglobin radical in cross-linking of myosin. *Free Radical Res.* **1995**, *22*, 215–227.
(19) Bhoite-Solomon, V.; Kessler-Icekson, G.; Shaklai, N. Peroxidative crosslinking of myosins. *Biochem. Int.* **1992**, *26*, 181–189.
(20) Ostdal, H.; Skibsted, L. H.; Andersen, H. J. Formation of long-lived protein radicals in the reaction between H_2O_2-activated metmyoglobin and other proteins. *Free Radical Biol. Med.* **1997**, *23*, 754–761.
(21) Xiong, Y. L. Protein oxidation and implications for muscle food quality. In *Antioxidants in Muscle Foods*; Decker, E., Faustman, C., Eds.; Wiley: Chichester, U.K., 2000; pp 85–111.
(22) Rowe, L. J.; Maddock, K. R.; Lonergan, S. M.; Huff-Lonergan, E. Influence of early postmortem protein oxidation on beef quality. *J. Anim. Sci.* **2004**, *82*, 785–793.
(23) Huff-Lonergan, E.; Lonergan, S. M. Mechanisms of water-holding capacity of meat: the role of postmortem biochemical and structural changes. *Meat Sci.* **2005**, *71*, 194–204.
(24) Lund, M. N.; Lametsch, R.; Hviid, M. S.; Jensen, O. N.; Skibsted, L. H. High-oxygen packaging atmosphere influences protein oxidation and tenderness of porcine longissimus dorsi during chill storage. *Meat Sci.* **2007**, *77*, 295–303.
(25) Smith, D. M. Functional and biochemical changes in deboned turkey due to frozen storage and lipid oxidation. *J. Food Sci.* **1987**, *52*, 22–27.
(26) Wan, L.; Xiong, Y. L.; Decker, E. A. Inhibition of oxidation during washing improves the functionality of bovine cardiac myofibrillar protein. *J. Agric. Food Chem.* **1993**, *41*, 2267–2271.
(27) Decker, E. A.; Xiong, Y. L.; Calvert, J. T.; Crum, A. D.; Blanchard, S. P. Chemical, physical, and functional properties of oxidized turkey white muscle myofibrillar proteins. *J. Agric. Food Chem.* **1993**, *41*, 186–189.
(28) Park, D.; Xiong, Y. L.; Alderton, A. L.; Ooizumi, T. Biochemical changes in myofibrillar protein isolates exposed to three oxidizing systems. *J. Agric. Food Chem.* **2006**, *54*, 4445–4451.
(29) Srinivasan, S.; Hultin, H. O. Chemical, physical, and functional properties of cod proteins modified by a nonenzymic free-radical-generating system. *J. Agric. Food Chem.* **1997**, *45*, 310–320.
(30) Ooizumi, T.; Xiong, Y. L. Biochemical susceptibility of myosin in chicken myofibrils subjected to hydroxyl radical oxidizing systems. *J. Agric. Food Chem.* **2004**, *52*, 4303–4307.
(31) Nauss, K. M.; Kitagawa, S.; Gergely, J. Pyrophosphate binding to and adenosine triphosphate activity of myosin and its proteolytic fragments. *J. Biol. Chem.* **1969**, *244*, 755–765.
(32) Wang, S. F.; Smith, D. M. Dynamic rheological properties and secondary structure of chicken breast myosin as influenced by isothermal heating. *J. Agric. Food Chem.* **1994**, *42*, 1434–1439.
(33) Expasy Proteomics Server MYH1_PIG. http://www. expasy. org/cgi bin/protparam1?Q9TV61@noft@ 2007.
(34) Andersen, H. J.; Skibsted, L. H. Kinetics and mechanism of thermal oxidation and photooxidation of nitrosylmyoglobin in aqueous solution. *J. Agric. Food Chem.* **1992**, *40*, 1741–1750.
(35) Nelson, D. P.; Kiesow, L. A. Enthalpy of decomposition of hydrogen peroxide by catalase at 25 °C (with molar extinction coefficient of H_2O_2 solution by UV). *Anal. Biochem.* **1972**, *49*, 474–478.
(36) Winterbourn, C. C. Oxidative reactions of hemoglobin. *Methods Enzymol.* **1990**, *186*, 265–272.
(37) Morzel, M.; Gatellier, P.; Sayd, T.; Renerre, M.; Laville, E. Chemical oxidation decreases proteolytic susceptibility of skeletal muscle myofibrillar proteins. *Meat Sci.* **2006**, *73*, 536–543.
(38) Stapelfeldt, H.; Bjerrum, K.; Skibsted, L. H. Ellman's reagent for determination of the heat treatment of milk powder. Improved analytical procedure based on stopped-flow kinetic study. *Milchwissenschaft* **1997**, *52*, 146–149.
(39) Smyth, D. G.; Nagamatsu, A.; Fruton, J. S. Some reactions of *N*-ethylmaleimide. *J. Am. Chem. Soc.* **1960**, *82*, 4600–4604.
(40) Smyth, D. G. Reaction of *N*-ethylmaleimide with peptides and amino acids. *Biochem. J.* **1964**, *91*, 589–595.
(41) Wada, R.; Kitabatake, N. β-Lactoglobulin A with *N*-ethylmaleimide-modified sulfhydryl residue, polymerized through intermolecular disulfide bridge on heating in the presence of dithiothretiol. *J. Agric. Food Chem.* **2001**, *49*, 4971–4976.
(42) Kroger-Ohlsen, M. V.; Ostdal, H.; Andersen, M. L. The effect of pH on the oxidation of bovine serum albumin by hypervalent myoglobin species. *Arch. Biochem. Biophys.* **2003**, *416*, 202–208.
(43) Miller, J. J.; Rice-Evans, C. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin. Sci.* **1993**, *84*, 407–412.
(44) Konczol, F.; Lorinczy, D.; Belagyi, J. Effect of oxygen free radicals on myosin in muscle fibres. *FEBS Lett.* **1998**, *427*, 341–344.
(45) Irwin, J. A.; Ostdal, H.; Davies, M. J. Myoglobin-induced oxidative damage: evidence for radical transfer from oxidized myoglobin to other proteins and antioxidants. *Arch. Biochem. Biophys.* **1999**, *362*, 94–104.
(46) Davis, J. S.; Buck, J.; Greene, E. P. The myosin dimer: an intermediate in the self-assembly of the thick filament of vertebrate skeletal-muscle. *FEBS Lett.* **1982**, *140*, 293–297.
(47) Reisler, E.; Cheung, P.; Borochov, N. Macromolecular assembly of myosin. *Biophys. J.* **1986**, *49*, 335–342.
(48) Wick, M. Filament assembly properties of the sarcomeric myosin heavy chain. *Poult. Sci.* **1999**, *78*, 735–742.
(49) Patel, R.; Svistuenko, D. A.; Darley-Usmar, V. M.; Symons, M. C. R.; Wilson, M. T. Redox cycling of human methaemoglobin by H_2O_2 yields persistent ferryl iron and protein based radicals. *Free Radical Res.* **1996**, *25*, 117–123.
(50) Barton, J. P.; Packer, J. E.; Sims, R. J. Kinetics of the reaction of hydrogen peroxide with cysteine and cysteamine. *J. Chem. Soc.* **1973**, 1547–1549.
(51) Stryer, L. Protein structure and function. In *Biochemistry*, 4th ed.; W. H. Freeman: New York, 1997; pp 17–46.
(52) Carlsen, U. C.; Skovgaard, I. M.; Skibsted, L. H. Pseudoperoxidase activity of myoglobin: kinetics and mechanism of the peroxidase cycle of myoglobin with H_2O_2 and 2,2-azino-bis(ethylbenzthiazoline-6-sulfonate) as substrates. *J. Agric. Food Chem.* **2003**, *51*, 5815–5823.
(53) Mikkelsen, A.; Skibsted, L. H. Kinetics of enzymatic reduction of metmyoglobin in realtion to oxygen activiation in meat products. *Z. Lebensm. Unters. Forsch.* **1992**, *194*, 9–16.
(54) Kroger-Ohlsen, M.; Skibsted, L. H. Kinetics and mechanism of reduction of ferrylmyoglobin by ascorbate and D-isoascorbate. *J. Agric. Food Chem.* **1997**, *45*, 668–676.

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