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REVIEWS

Myoglobin-Induced Lipid Oxidation. A Review

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An overview of myoglobin-initiated lipid oxidation in simple model systems, muscle, and muscle-based foods is presented. The potential role of myoglobin spin and redox states in initiating lipid oxidation is reviewed. Proposed mechanisms for myoglobin-initiated lipid oxidation in muscle tissue (pH 7.4) and meat (pH 5.5) are evaluated with the purpose of putting forward general mechanisms explaining present observations regarding the catalytic events.

Keywords: Myoglobin; heme; lipid oxidation; peroxidation; muscle-based foods

INTRODUCTION

Lipid oxidation leading to rancidity has been recognized since antiquity as a problem during the storage of fats and oils. Characteristic changes associated with the oxidative deterioration of vegetable oils and animal fats include the development of unpleasant tastes and odors, as well as changes in color, rheological properties, and solubility, and potential formation of toxic compounds such as 4-hydroxy-nonenal (*1*). The mechanisms by which unsaturated fatty acids react with molecular oxygen to undergo autoxidation, often referred to as lipid peroxidation, were established in the 1940s by Farmer, as reviewed by Gutteridge (*2*). Peroxidation has been thoroughly studied by food scientists, and it is well established that lipid oxidation is a major deteriorative reaction often resulting in a significant loss of quality. In contrast, the biological and medical aspects of lipid peroxidation have only been under investigation since the 1950s. However, subsequent anticipation of its pathological implications has resulted in extensive investigation, and many reviews have appeared (*3*−*7*).

The basic chemistry of the propagation of lipid peroxidation reactions has been known for years (*8, 9*), but the mechanism initiating this process is still uncertain and a matter of dispute, as spontaneous lipid radical formation or direct reaction of unsaturated fatty acids with molecular oxygen is thermodynamically unfavorable. Spin restriction, which prevents the direct addition of triplet state oxygen to singlet state unsaturated fatty acid molecules, can, however, be overcome, and oxidative degradation of lipids in biological systems and foods may be initiated by decomposition of endogenous species (H_2O_2, ROOH) or by radicals ($O_2^{\bullet-}$, ROO$^\bullet$, HO$^\bullet$, NO$^\bullet$, GS$^\bullet$) or by exogenous species ($^1O_2$, O_3), radicals ($^\bullet NO_x$, $SO_3^{-\bullet}$), and agents (UV, ionizing radiation, heat) (*10*, *11*). Nevertheless, most biological and food studies of lipid peroxidation involve transition metal ions (Fe^{n+}, Cu^{n+}, etc.), and it is generally accepted that iron is pivotal in catalyzing oxidative changes in tissues. Iron initiates lipid oxidation by generating free radicals capable of abstracting a proton from unsaturated fatty acids (*12, 13*). Different forms of iron have been proposed to initiate lipid oxidation causing pathological conditions in different tissue and resulting in oxidative deterioration in muscle foods (*14*−*17*). However, the relative contributions of different forms of iron, whether "free" or protein bound, heme or non-heme, oxidized or reduced, in catalyzing lipid peroxidation in most biological systems and in muscle-based foods have not been definitively assigned (*15*). Additionally, conflicting evidence about the roles of heme and non-heme bound iron in initiating lipid oxidation in muscle-based systems has been reported (*12, 15, 18*−*20*). However, discussion of these issues is not within the scope of this review and more detailed information pertaining to lipid oxidation in meats and in biological systems can be found in the excellent reviews by Kanner (*12*) and by Halliwell and Gutteridge (*6*), respectively. This paper aims at summarizing current information regarding the potential mechanisms by which muscle pigments induce lipid oxidation in muscle-based foods, supported by present knowledge of corresponding mechanisms in other biological systems.

Heme-Initiated Lipid Oxidation. The catalytic effect of heme compounds on the oxidative decomposition of polyunsaturated fatty acids was first described by Robinson in 1924 (*21*). Subsequently, a great deal of work has been performed regarding the bio-catalytic activity of these compounds. The wide distribution of heme compounds in biological systems and especially the high concentration of hemoglobin in red blood cells and myoglobin in tissues has, during the years, resulted in the assumption that heme-compound-catalyzed lipid peroxidation is a basic pathological reaction in vivo and a deteriorative reaction in muscle foods (*20, 22*−*24*). Muscle-pigment-initiated

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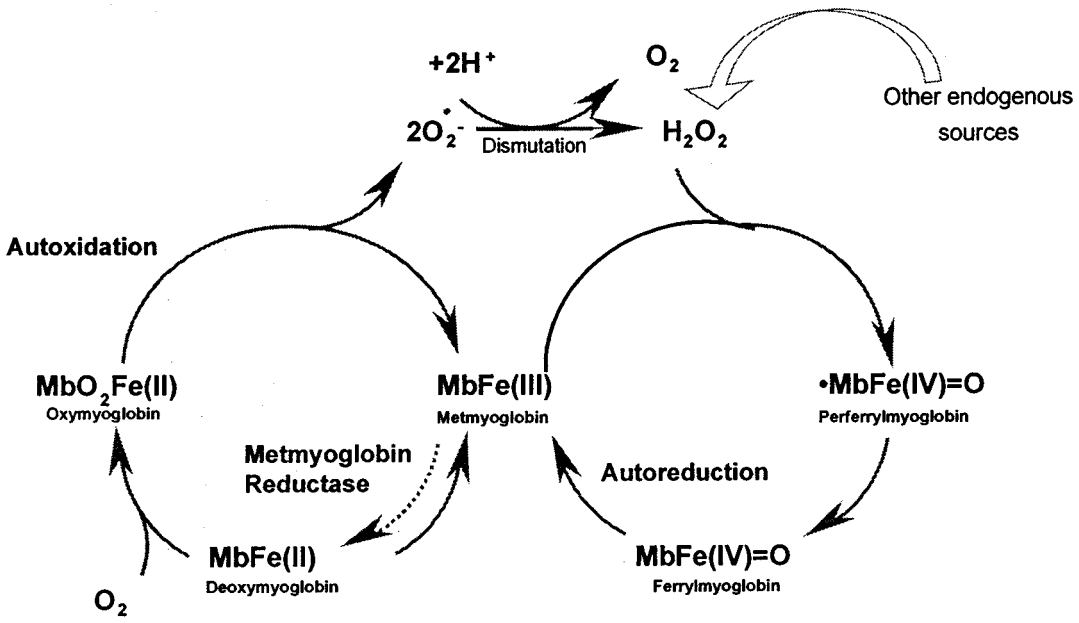
Scheme 1. Dynamic Conversion between the Different Myoglobin Pigments



lipid oxidation, leading to oxidative deterioration, has been extensively reported in meats, as lipid peroxidation in muscle foods is the reaction which often limits storage (*25*−*27*). In biological membranes, heme-protein-initiated lipid oxidation can cause impairment of membrane function, decrease fluidity, inactivate membrane-bound receptors and enzymes, and increase nonspecific permeability to ions such as Ca^{2+} (*2*, *6*, *28*).

Hemoglobin and Myoglobin in Muscle Tissue. The most abundant heme compounds found in vivo are myoglobin and hemoglobin. Their physiological function is to carry and distribute oxygen to the different tissues. Hemoglobin (Hb) is the main pigment in red blood cells and myoglobin (Mb) is the main pigment in the muscle cell. Both hemoglobin and myoglobin exist in different redox states, as illustrated for myoglobin in **Scheme 1**. Myoglobin is physiologically active in its deoxy-form (MbFe(II)) and oxy-iron(II)-state (MbFe(II)O_2). However, a continuous oxidation to the met-iron(III)-state (MbFe(III)) takes place in vivo, which, despite subsequent enzymatic reduction of the met-forms to the deoxy-forms gives an approximately 2−3% steady-state level of MbFe(III) in biological tissue and of HbFe(III) in blood (*6*). Furthermore, as shown in **Scheme 1**, MbFe(III) can be activated by reaction with hydrogen peroxide resulting in the formation of an unstable hypervalent, perferryl-iron(IV)-state (•MbFe(IV)), which is rapidly reduced to the more stable ferryl-iron(IV)-state (MbFe(IV)) (*29*−*31*). Even though the ferryl-iron(IV)-state is relatively stable, it is slowly reduced to the met-form. These hypervalent heme species have, during the past decade, received profound attention which has intensified after their recent detection in tissues and blood (*32*, *33*). Finally, disturbance of the globin structures of the deoxy-, oxy-, or met-forms of myoglobin and hemoglobin may result in either reversible or irreversible formation of low spin iron(II)-hemochromes or iron(III)-hemichromes (**Figure 1**). These denatured species have been observed in meats and in biological tissues, but have received little attention regarding their potential role and significance in lipid oxidation (*34*, *35*).

All the above-mentioned heme species may be found in meats (*35*, *36*). Differences in the ratio between myoglobin and hemoglobin between muscles and animal species have been reported. Myoglobin is the main pigment in beef, pork, and dark muscles of broilers, representing 70−90% of the total concentration of heme proteins (*35*, *36*−*38*). However, hemoglobin was found to be the only extractable pigment in white muscles of broilers (*38*) and accounts for approximately 50% to 60% in certain fish species (e.g., trout and mackerel) (*39*).

In contrast to the delicate balance between the deoxy-/oxy-forms and met-forms found in the living muscle, *post mortem* processes, and especially the pH fall, continuously inactivate the reductive enzyme systems and stimulate acid-catalyzed autoxidation of the iron(II) states to the iron(III) state of myoglobin in meats, resulting in accumulation of MbFe(III) (*40*−*42*).

Other heme proteins (e.g., cytochromes and ribonucleases) found in biological tissues display redox and spin characteristics similar to those of hemoglobin and myoglobin. Consequently, they must be expected to be able to participate in interactions with lipids equivalent to those described below for hemoglobin and myoglobin. Numerous studies report that some heme-containing peroxidases and cytochromes can initiate lipid peroxidation by mechanisms similar to those of myoglobin (*43*, *44*). However, the reaction rate might be different, as observed for hemoglobin in contrast to myoglobin (*45*). Additionally, the consequences of these interactions are not always known but are expected to be minor because of their low concentrations in tissues relative to those of hemoglobin and myoglobin (*36*).

The present review on myoglobin-induced lipid oxidation in muscle foods will include reports on hemoglobin-induced lipid oxidation where appropriate because of their similar nature. However, as mentioned above, some differences exist between these two heme proteins, their reactivity sometimes being quite different, although this will not always be stated in this review. Additionally, it should be noted that myoglobin or hemoglobin from different species can also differ in their reactivity and reaction mechanisms because of differences in their primary structures, however this aspect will not be discussed.

Iron(III) Myoglobin-Induced Lipid Oxidation. High-spin iron(III) myoglobin, commonly known as metmyoglobin (MbFe(III)), binds a molecule of water at the sixth coordination site of the heme iron (*46*), as illustrated in **Figure 1A**. Disturbance of the globin structure may alter the tertiary structure of the molecule and thereby expose the heme iron to unusual ligands (e.g., the distal histidine in the heme cavity,

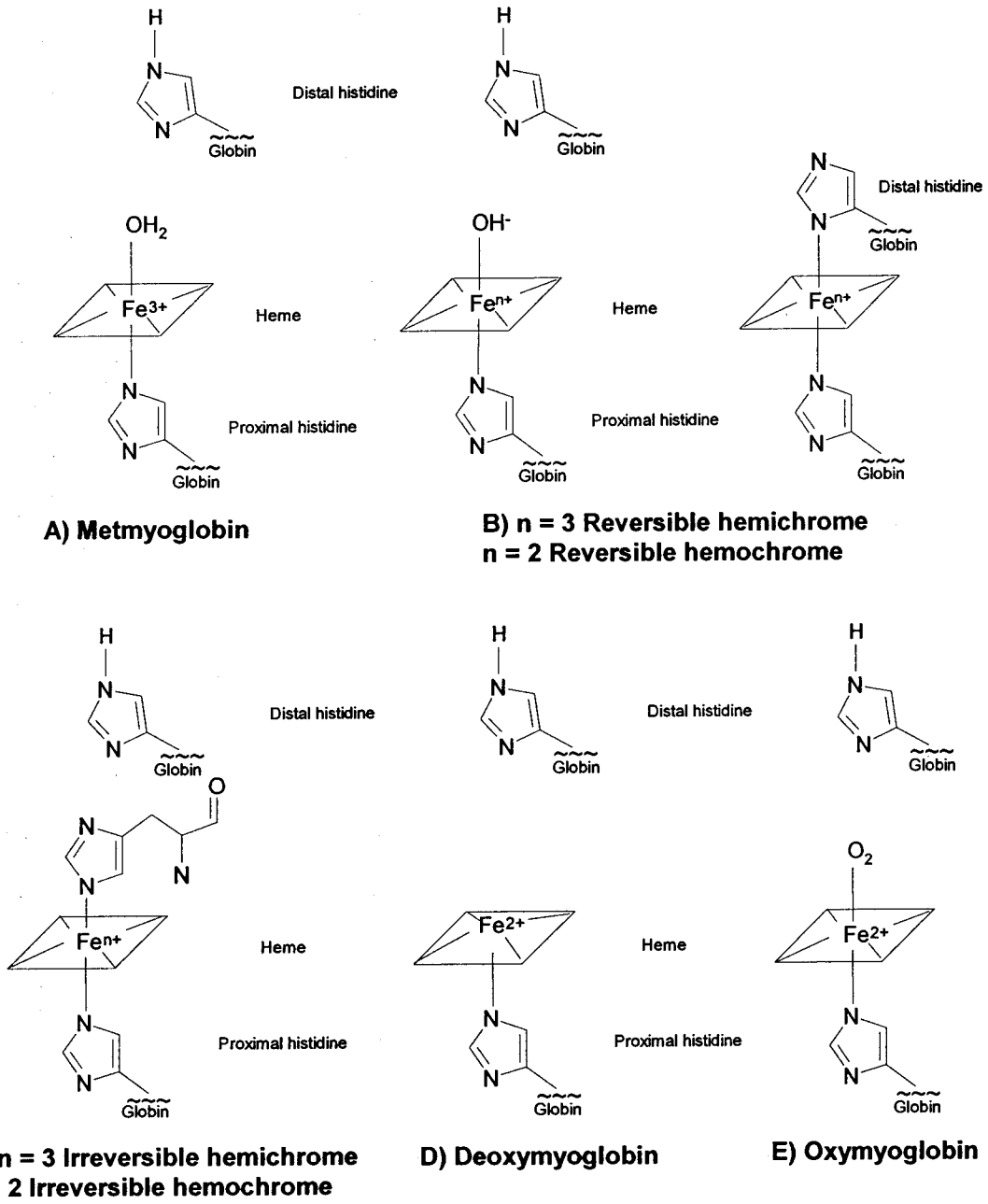


Figure 1. Different myoglobin species illustrated with their characteristic ligand bound to the 6th coordination site of the heme iron. (A) Metmyoglobin, water molecule bound at the ferric heme iron center; (B) reversible hemi-/hemochrome, hydroxyl ion bound at the ferric heme iron center; (C) irreversible hemi-/hemochrome, histidine residue bound at the ferric heme iron center; (D) deoxymyoglobin, no ligand at the ferrous heme iron center; (E) oxymyoglobin, oxygen molecule bound at the ferrous heme iron center.

exogenous amino acids as histidine and methionine, or a hydroxyl group) resulting in formation of the corresponding low-spin iron(III) myoglobin species better known as hemichromes (*47*). Hemichrome formation is either reversible (binding to the imidazole group of the distal histidine or hydroxyl ion) or irreversible (binding to the imidazole group of free histidine) depending on the type of ligand at the sixth coordination site of the iron and the extent of globin denaturation (**Figure 1B** and **C**, respectively). Hemichrome formation from iron(III) hemoglobin is the first step in the formation of Heinz bodies in red blood cells (*48, 49*), and an intermediate step in the heat denaturation of myoglobin in muscle foods (*35*).

Both MbFe(III) and HbFe(III) have been proposed to be involved in membranal lipid oxidation for decades, however, the exact nature of the catalyst giving rise to oxidative deterioration in biological systems is still a subject of great dispute. Most studies regarding the pro-oxidative activity of MbFe(III) and HbFe(III) in simple model systems at physiological pH have not been able to confirm any noticeable catalytic effect of either MbFe(III) or HbFe(III) (*23, 29, 30, 50*−*54*). However, several studies have stated that MbFe(III) is pro-oxidative only in the presence of peroxides (*55, 56*), which led Kanner to argued that at physiological pH MbFe(III) needs to be activated by H_2O_2 or lipid hydroperoxides to be an effective prooxidant (*29, 30*).

In contrast, pro-oxidative activity of these heme species has been demonstrated under more acidic conditions (pH 5.5−6.5), e.g., at inflammation sites and ischemic sites (*50, 57, 58*). The latter is supported by numerous investigations regarding heme-protein-initiated lipid oxidation in simple model systems resembling the conditions found in meat (*19, 52, 59*−*66*). Nevertheless, the pro-oxidative activity of MbFe(III) in meat

model systems has been argued because of the results of Sato and Hegarty (*67*), who were not able to demonstrate catalytic activity of meat pigments in their model system. Their data were subsequently supported by additional studies (*65, 68−70*). However, all these studies used a system in which muscle tissues were extensively washed. Consequently, these studies may have underestimated the pro-oxidative activity of MbFe(III) by washing away compounds such as hydroperoxides which could have been important for the pro-oxidative activity of MbFe(III) in situ (*12*). Formation of MbFe(III) is highly correlated to the extend of lipid oxidation in muscle foods (*25, 71−73*), and it is now generally accepted that MbFe(III) is a potential pro-oxidant at the pH found in fresh meat (between 5.3 and 6.2). Recent investigations emphasize that MbFe(III) is an effective pro-oxidant at acidic pH and in the presence of hydroperoxides (*66, 56, 74*) and hereby support the mechanistic theory for lipid-hydroperoxides dependent heme protein-catalyzed lipid oxidation proposed by Tappel as early as 1955 (*75*). Similar studies with hemoglobin also emphasize the importance of hydroperoxide in HbFe(III) initiated lipid oxidation (*76*).

In vivo, lipid hydroperoxides are the precursors of many physiological effectors including leukotrienes and prostaglandins via the arachidonic acid cascade (*77, 78*). However, evidences for continuous lipolysis in skeletal muscle (*79, 80*) and the release of the unstable arachidonic acid through hydrolysis of membrane phospholipid *post-mortem* (*81*) indicate that lipid hydroperoxides inevitably will rise during the conversion of muscle to meat. Especially released free fatty acid and reactive radicals, which are formed in the ischemic-like post-slaughter processes, must be expected to generate lipid hydroperoxides. However, no reports indicate the precise level of hydroperoxides in muscle tissues *post-mortem* despite the fact that hydroperoxides are present and rise in muscle after slaughter.

The lack of pro-oxidative activity of MbFe(III)/HbFe(III) found in model systems at physiological pH mentioned above has been proposed to be the result of an interaction between the heme proteins and free fatty acids (*51, 54*). Myoglobin has been postulated to possess specific electrostatic and hydrophobic binding sites for fatty acids (*82*). The specific sites for fatty acid binding have not been identified, however, the presence of at least two hydrophobic cavities in addition to the heme cavity have been suggested in MbFe(III) (*83*). Moreover, electrostatic association between the carboxylate ion of the fatty acid and the hemoglobin surface, accompanied by structural changes of the hemoglobin molecule, has been reported (*84*). Additionally, a recent investigation indicates that hemoglobin binding to phospholipids involves electrostatic and hydrophobic interactions, and that methemoglobin affects the structural and physicochemical parameters of the lipid−water interface (*85*). A similar interaction between free fatty acids and MbFe(III) has been suggested (*52, 86*), however, the importance of the charges in the interactions between fatty acids and heme proteins was only recently confirmed, as only linoleate (but not the corresponding linoleic acid methyl ester) was found to associate and effect structural changes in MbFe(III) (*54*). The interaction between MbFe(III) and long chain free fatty acids at physiological pH results in reversible formation of the low-spin iron(III) myoglobin species hemichrome (*54, 87, 88*). This hemichrome species is known to be a poor initiator of lipid oxidation (*54, 89*), as coordination of the distal histidine to the heme iron most likely hinders access of lipid hydroperoxides to the catalytic heme iron (*90*). Hemichrome formation at physiological pH in model systems containing long-chain free fatty acids seems, therefore, to be the most obvious mechanism for the noncatalytic activity of MbFe(III) and HbFe(III), while such a protective mechanism involving electrostatic association becomes impossible at lower pH, most probably due to different charge distribution on both the fatty acid and the heme protein.

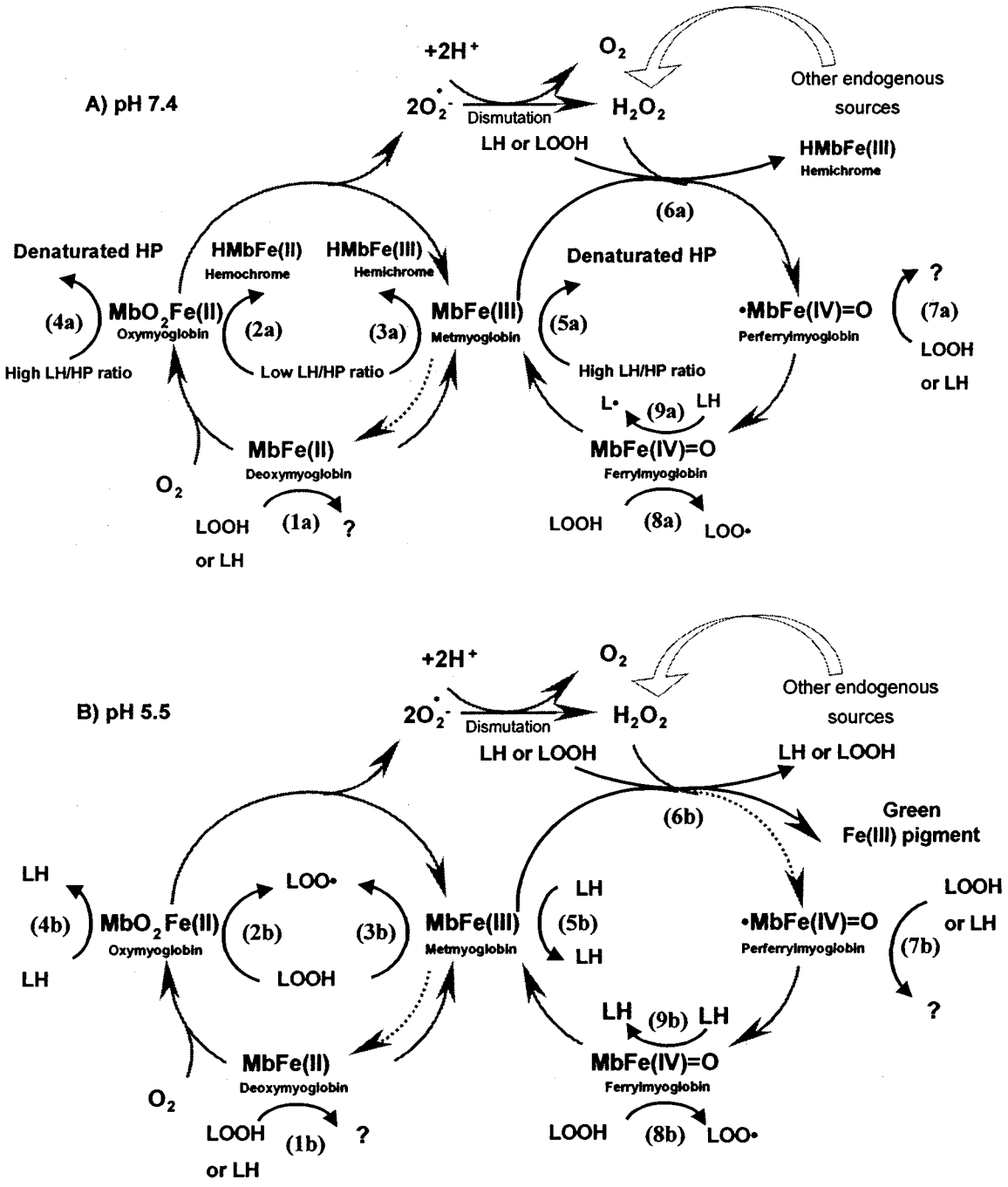
In recent years the vast interest in identifying a possible mechanism for postulated heme-protein-initiated lipid oxidation in living tissue has also brought into focus some earlier reports regarding both the antioxidative and pro-oxidative nature of iron (III) heme proteins in model systems (*43, 50, 86, 91, 92*). Maier and Tappel (*91*), using a simple linoleate system and a fixed methemoglobin concentration, reported that when the linoleate concentration dropped below a certain concentration a prolonged induction period appeared. Lewis and Wills (*92*) confirmed similar anti- or pro-oxidative activity of MbFe(III) depending of the heme/lipid ratio in an identical model system. These data have subsequently been confirmed by others (*23, 50, 55, 86*).

The so-called antioxidative activity of the different heme proteins was found at a linoleate-to-heme ratio ranging from 50 to 200 and can most probably be attributed to the above-mentioned interaction between the heme proteins and the long-chain free fatty acids resulting in noncatalytic hemichrome species. The pro-oxidative activity of the heme proteins is only observed when the linoleate-to-heme ratio becomes higher than 250 (*50*). A recent study by Baron et al. (*93*) reported that at a linoleate-to-heme ratios of 300 or more, metmyoglobin immediately denatures and results in exposure or release of the heme group to the environment that instantly initiates hematin-induced lipid peroxidation in the system. This make us suggest that the observed MbFe(III) induced lipid oxidation at physiological pH in reality ought to be interpreted as hematin-induced lipid oxidation. Such a mechanism is also in agreement with the observed formation of hemichrome during the interaction between unstable hemoglobin and liposomes, and subsequent transfer of the heme from the globin to the lipid phase resulting in initiation of lipid oxidation (*94−97*). Similar initiation of lipid oxidation may be of relevance in processed meat, e.g., in fermented products with high lipolysis or products with high fatty acids-to-pigment ratio. However, these interactions between heme proteins and lipids deserved further attention, and especially interactions with triglycerides and phospholipids, which are the major lipids components in processed meat. This area has until now not received any attention and needs to be further explored if a better understanding of oxidative deterioration of muscle foods containing a high percentage of lipids is to be achieved.

In summary, available data regarding MbFe(III) initiated lipid oxidation at physiological pH suggest that in the presence of lipids MbFe(III) can undergo a rapid neutralization due to formation of the noncatalytic hemichrome pigment. However, further denaturation of the heme protein due to a high lipophilic environment may result in heme release or further exposure of the heme group to the surrounding lipids and thereby induce lipid peroxidation. In contrast, at acidic pH, MbFe(III) was found to be able to initiate lipid oxidation in a lipid hydroperoxide-dependent mechanism.

Iron (II) Myoglobin-Induced Lipid Oxidation. The physiologically active myoglobin species are the purple high-spin iron (II) myoglobin (deoxymyoglobin (MbFe(II)), which has the sixth coordination site of the heme iron vacant, and the bright cherry-red low-spin oxy-iron (II) myoglobin (oxymyoglobin ($MbFe(II)O_2$)), which bind a molecule of oxygen at the sixth coordination of the heme iron, due to its high affinity for oxygen (**Scheme 2D**, **2E**, respectively). Like MbFe(III), disturbance of the globin structure can result in binding of an unusual ligand

Scheme 2. Redox Cycle of the Different Species and Their Proposed Contribution to Lipid Oxidation/Peroxidation: (A) at Physiological pH (7.4); and (B) at pH of Relevance for Meat and Meat Products (5.5)



at the sixth coordination of the heme iron and induce the formation of a low-spin iron (II) species, known as hemochromes (**Figure 1B** and **C**). Hemochromes differ from hemichromes exclusively in the oxidation state of the iron center, the former being in its oxidation state II. Hemochromes, like hemichromes, can be found either reversible or irreversible.

Only a few studies have reported pro-oxidative activity of $MbFe(II)O_2$ in biological systems. Hogg et al. (*56*) showed that $MbFe(II)O_2$ can promote oxidative modification of LDL as also proposed by others (*98*). Contrary to this, Galaris et al. (*87*) showed visible absorption spectral change of $MbFe(II)O_2$ upon incubation with linoleic acid at physiological pH, which can be attributed to formation of the noncatalytic low-spin myoglobin derivative, hemochrome, also reported for $HbFe(II)O_2$ (*89*).

For many years, it was assumed that MbFe(III) was the only heme protein species responsible for the initiation of lipid oxidation in muscle-based foods. This has been the basis of the general assumption that maintenance of the bright cherry-red color of meat can delay oxidative deterioration of muscle-based foods (*71*, *99*). However, whether the oxidation state of the iron is of any significance in the initiation process of lipid oxidation by heme proteins has been questioned (*100*). Subsequently, $MbFe(II)O_2$ has been shown to initiate lipid oxidation as effectively as MbFe(III) even though the mechanism by which $MbFe(II)O_2$ initiates lipid oxidation still is unclear (*70*, *101*). Faustman and co-workers report a high correlation between $MbFe(II)O_2$ oxidation and lipid oxidation both in microsomes and liposomes and believe that $MbFe(II)O_2$ oxidation and lipid oxidation are coupled (*102*, *103*). It should be emphasized that

the pro-oxidative activity of MbFe(II)O_2, as such, is difficult to assess because of continuous autoxidation of MbFe(II)O_2 to MbFe(III). This becomes even more complicated in meats, as the low pH found in most muscle-based foods accelerates autoxidation of MbFe(II)O_2 to MbFe(III) (*40*). Moreover, Chan et al. (*101*) showed that addition of catalase in a liposome system reduced the pro-oxidative activity of MbFe(II)O_2, indicating that H_2O_2 produced during MbFe(II)O_2 autoxidation may activate MbFe(II)O_2 to high oxidation state myoglobin species, which are believed to possess a more pronounced pro-oxidative activity. Recent studies show, however, that this eventually formed high oxidation state myoglobin, ferrylmyoglobin, has pro-oxidative activity comparable to that of MbFe(II)O_2 under conditions found in muscle-based systems (*66*). Most studies show that both MbFe(II)O_2 and MbFe(III) are pro-oxidants, and the difference observed in their ability to initiate lipid oxidation might be trivial as stated by Hirano and Olcott (*55*). This was confirmed in a linoleic acid emulsion model system simulating the conditions found in muscle-based foods (*66*) and by a recent study using a similar model system (*104*).

The pro-oxidative activity of deoxymyoglobin in biological systems including muscle foods has not been investigated. This is mainly due to the fact that MbFe(II) initiated lipid oxidation demands strict anaerobic conditions, to exclude MbFe(II)O_2 initiated lipid oxidation and the subsequent propagation of lipid oxidation. However, a recent study suggested that, in contrast to methemoglobin, deoxyhemoglobin was able to initiate lipid oxidation even at low lipid hydroperoxide concentrations (*104*). They postulate a potential role for deoxyhemoglobin as initiator of lipid oxidation in a lipid hydroperoxide independent mechanism. Further work is needed to confirm this finding in order to elucidate by which mechanism deoxymyoglobin initiates lipid oxidation.

In summary, under physiological conditions, as reported for MbFe(III), MbFe(II)O_2 is not expected to be pro-oxidative. At low lipid concentrations MbFe(II)O_2-induced lipid oxidation can be retarded because of conversion of MbFe(II)O_2 to the noncatalytic low-spin derivative, hemochrome. However, in more complex systems with high lipid and/or protein concentrations, further denaturation of MbFe(II)O_2 may occur, which subsequently might give rise to indirect myoglobin-catalyzed lipid oxidation through heme exposure followed by hematin-induced lipid oxidation. In muscle-based foods, MbFe(II)O_2 seems to be just as effective as MbFe(III) in the initiation of lipid peroxidation by the well-described hematin-induced lipid peroxidation mechanism (*75*).

Iron (IV) Myoglobin-Induced Lipid Oxidation. The reaction between hydrogen peroxide and MbFe(III) resulting in the formation of a red pigment has been known for decades (*105, 106*). During this interaction, Gibson et al. (*107*) observed the production of free radicals and postulated that oxidation occurs in the globin part of the heme protein. As mentioned earlier, Kanner and Harel (*29, 30*) indicated that hydrogen peroxide activation of MbFe(III) (also called activated-myoglobin), was a necessary step in the conversion of MbFe(III) to a pro-oxidant. Further studies have shown that interaction between MbFe(III) and hydrogen peroxide is a complex mechanism, resulting in the generation of two distinct hypervalent myoglobin species, •MbFe(IV)═O and MbFe(IV)═O (eq 1) (*108, 109*).

$$\underset{}{\text{MbFe(III)}} + H_2O_2 \rightarrow \underset{\text{perferrylmyoglobin}}{\text{•MbFe(IV)═O}} \rightarrow \underset{\text{ferrylmyoglobin}}{\text{MbFe(IV)═O}} \quad (1)$$

The formation of these hypervalent myoglobin species proceeds via a direct transfer of two oxidation equivalents, from hydrogen peroxide to metmyoglobin, giving perferrylmyoglobin (•MbFe(IV)═O) (110). One equivalent is located at the iron center forming an oxoferryl complex (Fe(IV)═O), while the other equivalent is suggested to be rapidly transferred from the heme to an amino acid of the globin, giving a protein radical (*108*). Perferrylmyoglobin is a transient species with a very short half-life and autoreduces rapidly to the more stable ferrylmyoglobin (MbFe(IV)═O) by a poorly understood mechanism (*111*). Ferrylmyoglobin is a relatively stable species which slowly is reduced back to MbFe(III) at physiological pH but with an increasing rate at decreasing pH due to an acid-catalyzed process (*112−113*).

The reactivities of MbFe(III) and HbFe(III) toward H_2O_2 have been reported to be profoundly different (*45*). MbFe(III) has been shown to react very quickly and to require only moderate excess of H_2O_2, whereas HbFe(III) requires a 10-fold excess of H_2O_2 and a 30-min reaction to form the perferryl species. These perferryl species showed large differences in their stabilities with a half-life of 50 to 280 ms for •MbFe(IV)═O and a half-life of 50 s for •HbFe(IV)═O. Additionally, MbFe(IV)═O showed a considerably more pronounced pro-oxidative activity when compared to HbFe(IV)═O.

The iron(II) myoglobin species, MbFe(II) and MbFe(II)O_2, can likewise react with hydrogen peroxide resulting in formation of ferrylmyoglobin by direct two-electron oxidation of these iron(II) myoglobin species (*109, 114*), as shown for oxymyoglobin in eq 2, without formation of the transient •MbFe(IV)═O. A similar mechanism has been reported for the interaction of oxyhemoglobin with hydrogen peroxide (*115*).

$$\underset{\text{oxymyoglobin}}{\text{MbFe(II)}O_2} + H_2O_2 \rightarrow \underset{\text{ferrylmyoglobin}}{\text{MbFe(IV)═O}} + H_2O_2 + O_2 \quad (2)$$

In the literature the differentiation between ferryl- and perferrylmyoglobin is not clear, and many studies related to the pro-oxidative activity of these hypervalent myoglobin species do not make a distinction between the two. This gave rise to a number of publications in which the reactive species under investigation was not obvious and has led to a general confusion regarding their pro-oxidative activity. The term H_2O_2-activated myoglobin has been used extensively even though this includes both the protein radical and the ferryl species. We have assumed that in these studies the pro-oxidative activity is then assigned to both species.

The interaction between lipid hydroperoxides and MbFe(III) has been studied to a much lesser extent than the interaction between MbFe(III) and hydrogen peroxide, even though it is believed to proceed via the formation of hypervalent myoglobin species (*113, 116−118*). However, opposing evidence exists in the literature. Incubation of MbFe(III) and purified linoleic acid hydroperoxides has been suggested to generate MbFe(IV)═O in a one-electron redox reaction without the formation of •MbFe(IV)═O (*113, 116, 117*). However, Maiorino et al. showed that MbFe(III) incubation with phospholipids hydroperoxides did not result in the formation of ferryl nor of perferryl myoglobin (*118*). In contrast to these reports, interaction between MbFe(III) and organic peroxides (i.e., ethyl hydroperoxide or *tert*-butyl hydroperoxide) clearly indicates the formation of both oxoferryl species •MbFe(IV)═O and MbFe(IV)═O (*74, 119*). Much more work is needed to elucidate these interactions to put forward more explicit reaction mechanisms.

Perferrylmyoglobin. The ability of •MbFe(IV)═O to initiate lipid oxidation and to abstract an allylic hydrogen atom from fatty acids has been suggested (eq 3) (*29, 30, 52, 120*).

$$^{\bullet}\text{MbFe(IV)=O} + \text{LH} \rightarrow \text{MbFe(IV)=O} + \text{L}^{\bullet} + \text{H}^{+} \quad (3)$$

Moreover, Davies (*109*), in a simple linoleate emulsion model system and at pH 7.4, showed that the intensity of the characteristic $^{\bullet}$MbFe(IV)=O electron spin resonance (ESR) signal is reduced approximately 90% compared to the signal in an equal system without linoleate, and they concluded that a direct interaction between $^{\bullet}$MbFe(IV)=O and the lipid occurred, initiating lipid oxidation. However, a recent study in an equal model system has shown that addition of MbFe(III) to a reaction mixture containing linoleate and H_2O_2 does not result in the presumed perferryl species. Instead, MbFe(III) is converted to its low-spin compound, hemichrome (*54*), which explains the poor formation of the characteristic $^{\bullet}$MbFe(IV)=O ESR signal seen by Davies (*108*) rather than the proposed oxidation of unsaturated fatty acids. Hemichrome cannot be activated subsequently to any hypervalent myoglobin species by excess H_2O_2 and initiate lipid oxidation, as the linkage between the distal histidine residue and the heme iron center prevents the access of hydrogen peroxide to the iron center (*121*). Activation of MbFe(III) by H_2O_2 and immediate addition to membranal lipids have been shown to promote lipid oxidation (*29*), therefore suggesting that $^{\bullet}$MbFe(IV)=O was an effective prooxidant. However, this applies only if MbFe(III) is activated by peroxides to yield $^{\bullet}$MbFe(IV)=O prior to interaction with the lipid components.

Despite the limiting evidence for a pro-oxidative activity of $^{\bullet}$MbFe(IV)=O in lipid systems, MbFe(III)/H_2O_2 has been shown to oxidize a number of organic molecules, e.g., styrene (*122*), chlorpromazine (antipsychotic) (*123*), and crocin (*124*). Furthermore, $^{\bullet}$MbFe(IV)=O has been shown to induce both intra- and intermolecular protein cross-linking (*125*−*130*). These reactions proceed via tyrosine residue(s) in the globin part of the molecule (*131*). Also, recent investigations have shown that $^{\bullet}$MbFe(IV)=O is able to transfer its protein radical to other proteins and thereby generate reactive secondary protein radicals with extremely long half-lives (*132*−*134*). The reactivity of these protein radicals against lipids has not been extensively investigated. However, a recent report by Østdal and co-workers clearly indicates for the first time that such protein radicals are able to initiate lipid peroxidation in polyunsaturated free fatty acids emulsions as well as promote protein oxidation (*135*). Not only the transfer of the protein radical on $^{\bullet}$MbFe(IV)=O to other proteins, but also the direct interaction between protein radicals and lipids, may account for a potential oxidation initiation mechanism in lipoproteins or lipid−protein mixed systems which are present in all biological systems and deserve further attention in the future. However, a potential antioxidative process for such protein radical transfer has not yet been excluded.

The generation of $^{\bullet}$MbFe(IV)=O by interaction of MbFe(III) with H_2O_2 at acidic pHs (5. 5−6. 5) has been shown to be negligible (*136*). This was confirmed in a recent study (*66*), where it was found that H_2O_2 activation of MbFe(III) under acidic conditions resulted in $^{\bullet}$MbFe(IV)=O formation, which underwent a rapid intramolecular rearrangement leading to cross-linkage between the prosthetic heme group and the globin rather than interacting with lipids under conditions expected to be found in meats (*66*). The heme protein species formed under these conditions, which has a characteristic green color, has been described in other studies (*106, 137*). Even though this green adduct has been shown to oxidize NADH (138) and give rise to cellular damage (*139*) it was not able to initiate lipid oxidation in a simple model system (*66*).

Ferrylmyoglobin. Ferrylmyoglobin has been reported to be responsible for the oxidation of a variety of substrates including proteins, ascorbic acid, tocopherols, glutathione, β-carotene, and Trolox (*52, 88, 140*−*142*). However, little information is available regarding the ability of MbFe(IV)=O to initiate lipid oxidation in muscle foods. Under conditions similar to those found in muscle foods, MbFe(IV)=O has been shown to initiate lipid oxidation in a lipid hydroperoxide dependent mechanism (*56, 66*). However, under the conditions found in fresh meat (pH 5.5−5.8) MbFe(IV)=O autoreduces rapidly to MbFe(III), and it cannot be excluded that the observed pro-oxidative activity is a result of MbFe(III) initiated lipid oxidation. Nevertheless, under physiological conditions (pH 7.4), MbFe(IV)=O has also been shown to initiate lipid oxidation under conditions where MbFe(III) is not a pro-oxidant, and these findings confirm the ability of MbFe(IV)=O to initiate lipid oxidation (*54*). Similarly, Rao et al. (*88*) reported that under physiological conditions, MbFe(IV)=O is a strong pro-oxidant, able to abstract a hydrogen atom from fatty acids, with subsequent stereospecific addition of oxygen (*87*).

The pro-oxidative activity of MbFe(IV)=O is independent of pH and of lipid concentration (*54, 66, 93*). Under physiological conditions, and in the presence of fatty acids, MbFe(IV)=O is not converted to nonpro-oxidative hemichrome (*54*). Increasing the lipid/heme protein ratio did not affect the pro-oxidative activity of MbFe(IV)=O, nor did it result in any drastic heme protein denaturation, as observed for MbFe(III) (*93*). On the basis of these observations, its seems that, irrespective of the pH and of the lipid/heme protein ratio, MbFe(IV)=O is able to initiate lipid oxidation via the same mechanisms. This may be of importance in biological systems if MbFe(III) interacts, initially, with hydrogen peroxide rather than with the lipid components, generating MbFe(IV)=O, which can subsequently initiate lipid oxidation. Moreover, the presence of reducing agents with the capacity to convert MbFe(IV)=O to MbFe(III) may be a crucial determinant of oxidative damage in biological systems. A large number of reducing agents present in muscle tissues (i.e., glutatione, NADH, ascorbate) are capable of reducing MbFe(IV)=O to MbFe(III) as first reported by Kanner (*12*) and later confirmed in other studies (*140, 141, 143*). These pathways of MbFe(IV)=O reduction are suggested to be of importance in muscle tissue (*145, 146*). However, in meat systems, the level of reducing agents able to convert MbFe(IV)=O to MbFe(III) is continuously depleted post-slaughter because of a diverse number of oxygen-consuming processes known to depend on the physiological status of the muscle *ante mortem* (*147*), which enables MbFe(IV)=O accumulation. Moreover, conversion of MbFe(IV)=O to MbFe(III) by reducing agents may be of no significance, as MbFe(III) and MbFe(IV)=O have been shown to have equivalent pro-oxidative activities in a linoleic acid model system simulating the conditions in meat (*66*).

In summary, under the conditions found in raw muscle food perferrylmyoglobin is not expected to be able to initiate lipid oxidation. Interaction between MbFe(III) with hydrogen peroxide, to yield perferrylmyoglobin, results in an intramolecular electron-transfer giving a heme-globin cross-linkage and generating a non-pro-oxidative green iron (III) heme pigment. The reactivity of perferrylmyoglobin toward lipids under physiological conditions is difficult to assess because of competitive conversion of MbFe(III) to hemichrome or to perferrylmyoglobin in favor of hemichrome, which prevents significant lipid oxidation. However, it seems that perferrylmyoglobin can effectively transfer its radical to other proteins, which may, subsequently, induce lipid oxidation. In contrast, ferrylmyoglobin is expected to be an effective pro-oxidant under the

conditions found in muscle food, as well as under physiological conditions. However, ferrylmyoglobin formation in muscle tissues will be determined by H_2O_2 and lipid hydroperoxide production and availability, and its potential to oxidize lipids will also be dependent on the concentration of reducing agents and their compartmentalization in the muscle cells.

SYNOPSIS

Mechanisms of myoglobin-induced oxidation/peroxidation of polyunsaturated fatty acids are still a matter of dispute. The reviewed data suggest that oxidation/peroxidation induced by the presence of myoglobin is highly pH dependent. Consequently, the mechanisms observed under physiological conditions in vivo, are profoundly different from those occurring in meats. **Scheme 2A** and **2B** give a representation of the redox cycle of myoglobin species and their supposed contribution to the oxidation of unsaturated fatty acids (LH) and breakdown of lipid hydroperoxides (LOOH) at physiological pH value (7.4) and at pH values found in fresh meat (5.5), respectively. Hardly any evidence exists regarding the ability of deoxymyoglobin to initiate lipid oxidation or peroxidation at physiological pH (1a) or at more acidic pH values (1b). Consequently, research needs to be initiated to answer these issues. In contrast, at physiological pH, oxygenated myoglobin does not seem to play any role in initiating lipid oxygenation, as oxymyoglobin is transformed to an nonactive hemochrome species in the presence of low levels of unsaturated free fatty acid with respect to heme protein (LH/HP) (2a). Likewise, oxymyoglobin, and under similar conditions metmyoglobin, is transformed into a nonreactive species hemichrome. However, at high unsaturated fatty acid/heme protein (LH/HP) ratios both oxymyoglobin and metmyoglobin are denatured (4a and 5a) resulting in exposure of the heme group to the environment, which gives rise to a hematin-induced peroxidation mechanism. Such processes might contribute to the poor stability of meat and meat products with high fat content. The conversion of potentially pro-oxidative heme protein to nonreactive species deserves further attention, and especially the interactions between heme proteins and triglycerides/phospholipids. Additionally, more work is needed to further assess the importance of denatured heme proteins in initiating lipid peroxidation/oxidation. At pH values of relevance for meat and meat products, both metmyoglobin and oxymyoglobin have been shown to be major initiators of lipid oxidation (2b and 3b) and peroxidation (4b and 5b). However, perferrylmyoglobin, which has been suggested to be a main candidate as an initiator of lipid oxidation/peroxidation both at physiological pH and at more acidic pH values, has not yet been proven to be involved in lipid oxidation/peroxidation processes (7a and 7b). Indeed, in the presence of lipid and hydrogen peroxide and under physiological conditions, metmyoglobin is preferentially converted to its corresponding hemichrome (6a) and little perferrylmyoglobin is formed. Similarly, under more acidic conditions, metmyoglobin, in its presence of hydrogen peroxide, is converted to a green pigment by intramolecular cross-linking and little perferrylmyoglobin can be observed (6b). The major problems assessing perferrylmyoglobin pro-oxidative activity lie in the transient radical character of the myoglobin derivative and factors influencing its formation and stability. However, it has been reported that perferrylmyoglobin is able to transfer its radical to other proteins and biomolecules, indication that this area deserves further attention with regard to perferrylmyoglobin-induced oxidation. An in-depth knowledge of the relationship between lipid and protein oxidation might reveal important information regarding the oxidative stability of meat and meat products. In contrast to perferrylmyoglobin, the transient ferrylmyoglobin species has proved to be a strong prooxidant, able to initiate lipid oxidation/peroxidation irrespective of the pH (8a, 8b and 9a, 9b).

Surprisingly, much of the data obtained in simple model systems regarding myoglobin induced lipid oxidation/peroxidation is compatible with empirical data observed in vivo, ex vivo, and in muscle foods. However, the relative importance of the different myoglobin species in the initiation of lipid oxidation/peroxidation in biological systems, including muscle-based foods, needs to be systematically determined, as this information is essential for both devising appropriate and adequate measurements of potential damage and preventing it in tissue- and muscle-based food systems.

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