

## MYOGLOBINS – THE LINK BETWEEN DISCOLORATION AND LIPID OXIDATION IN MUSCLE AND MEAT

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Aerobic metabolism changes rapidly to glycolysis post-mortem resulting in a pH-decrease during the transformation of muscle into meat affecting ligand binding and redox potential of the heme iron in myoglobin, the meat pigment. The “inorganic chemistry” of meat involves (i) redox-cycling between iron(II), iron(III), and iron(IV)/protein radicals; (ii) ligand exchange processes; and (iii) spin-equilibria with a change in coordination number for the heme iron. In addition to the function of myoglobin for oxygen storage, new physiological roles of myoglobin are currently being discovered, which notably find close parallels in the processes in fresh meat and nitrite-cured meat products. Myoglobin may be characterized as a bioreactor for small molecules like O<sub>2</sub>, NO, CO, CO<sub>2</sub>, H<sub>2</sub>O, and HNO with importance in bio-regulation and in protection against oxidative stress in vivo otherwise affecting lipids in membranes. Many of these processes may be recognised as colour changes in fresh meat and cured meat products under different atmospheric conditions, and could also be instructive for teaching purposes.

Keywords: myoglobin complexes; heme iron; oxidative processes.

## INTRODUCTION

The primary physiological role of the small iron-containing globular protein, myoglobin (Mb), in mammals has been considered to maintain oxygen supply in skeletal muscles and other muscle tissue, where Mb acts in oxygen storage and the facilitated diffusion of molecular oxygen.<sup>1</sup> New studies, however, strongly support other physiological roles of Mb in mammals.<sup>2-4</sup> Mb has served as a model molecule for other larger and more complex metalloproteins over the years, and experimental findings for Mb have been useful for generalization in relation to molecular structure,<sup>5</sup> biophysics and mechanism of enzyme activation.<sup>6,7</sup> Apart from the physiological function of Mb and its role as a model for other macromolecules, the chemistry of Mb is important in food technology as the colour of both fresh meat and meat products depends on the redox status of the heme iron center.<sup>8,9</sup>

Mb is a small globular protein with about 150 amino acid residues and molecular weight of approx. 17 kD.<sup>10</sup> A porphyrin ring, protoporphyrin IX, is partly buried in the interior of Mb and a central iron atom is coordinated to this prosthetic group forming a heme moiety, which constitutes the active site of the molecule. The protein backbone and the prosthetic group are attached by a single coordinative bond between the His94 residue and the heme iron, while hydrophobic interactions between vinyl side chains of the prosthetic group and hydrophobic amino acid residues in the interior of Mb also helps to stabilize the association.

This present review covers mainly the chemistry behind the colour and colour changes of fresh meat and meat products during processing and storage. The “inorganic chemistry” of meat will be discussed in relation to the coordination properties of the central heme iron atom in Mb and its redox chemistry. The redox chemistry of Mb and other heme proteins will also be discussed in relation to oxidative pro-

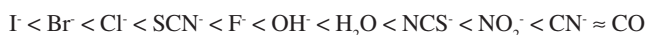
cesses occurring in muscle during transformation to meat and during meat curing, and these processes may cause deterioration of freshness and initiate lipid oxidation leading to rancidity.

## CHEMISTRY OF HEME IRON CENTRE IN MYOGLOBINS

Under most physiological conditions, the iron atom in Mb and other heme proteins exists in either the ferrous(II) or the ferric(III) state with six or five electrons in the 3d orbital available for bonding (d<sup>6</sup> or d<sup>5</sup> electron configuration).<sup>9</sup>

The relatively high numbers of electrons present in the 3d orbital makes several different electronic configurations possible for the iron complex with small differences in electronic energy.<sup>8</sup> Low spin Fe<sup>II</sup> complexes are diamagnetic, while all other spin states of both Fe<sup>II</sup> and Fe<sup>III</sup> contain at least one unpaired electron.

Ligands are ordered according to increasing “field strength” in what is known as the spectrochemical series depending on their effect on the splitting ( $\Delta$ ) between t<sub>2g</sub> and e<sub>g</sub> orbitals:<sup>11</sup>



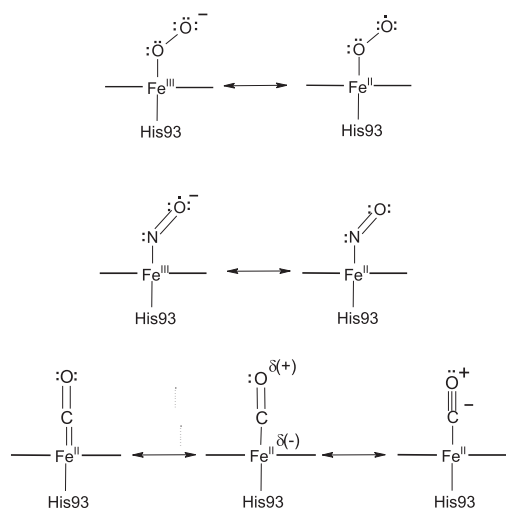
Thus, weak field ligands such as the halides favour high spin states as these ligands only participate in  $\sigma$ -bonding, while strong field ligands like O<sub>2</sub>, NO and CN<sup>-</sup> favour low spin state complexes with electron pairing of the d orbitals of iron.<sup>9,12</sup> Thus, the bond formation between the central metal and a strong field ligand may be considered to consist of two components: i) donation of  $\sigma$ -type electron density from the ligand to the central metal, and ii) donation of electron density from the d orbitals of the metal into the  $\pi^*$ -antibonding of the ligand referred to as “back-bonding”.<sup>13</sup> Regarding to physiologically relevant ligands, it has been pointed out that the rate of ligand association to heme iron(II) follows the order of the  $\pi$ -electron accepting abilities of the ligands, e.g. NO > O<sub>2</sub>  $\geq$  CO.<sup>8</sup>

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## METAL LIGAND ELECTRONIC DISTRIBUTION

Myoglobin heme iron has strong affinity for  $\pi$ -accepting ligands such as NO.<sup>13</sup> Diatomic ligands like NO and O<sub>2</sub> exist in different resonance forms. These ligands form complexes with transition metals that exhibit different electronic distributions, which besides the number of electrons in the complex are also affected by a varying degree of electronegativity of the diatomic ligands. Figure 1 shows examples of bonding geometry and electronic resonance forms for complexes of Fe<sup>II</sup> heme and three diatomic ligands of physiological importance. The different interactions between heme iron and ligands are due to differences in the dipole moment and other electronic properties. Thus, distribution of charge along the ligand or ionic character of the ligand greatly affects the ability to participate in hydrogen bonding with the amino acid residue, His64, in the distal heme pocket of Mb, which accordingly also influences the stabilization of the metal ligand complex.

The notation for nitrosyl complexes incorporating bonding, bond geometry and reactivity developed by Enemark and Feltham has generally been accepted to predict the bonding geometry.<sup>12</sup> The d orbital electrons of the metal and the  $\pi^*$  orbital electrons from the ligand together are counted as the overall number of electrons in the complex, and the nitrosylated complexes of Fe<sup>III</sup> or Fe<sup>II</sup> Mb can be written as {Fe(NO)}<sup>6</sup> [Fe (d<sup>6-1</sup>) + 1  $\pi^*$  electron] and {Fe(NO)}<sup>7</sup> [Fe (d<sup>6</sup>) + 1  $\pi^*$  electron], respectively, and used to predict the Fe-N-O bond angle, as linear for the Fe<sup>III</sup> complex and bent for the Fe<sup>II</sup> complex, respectively.

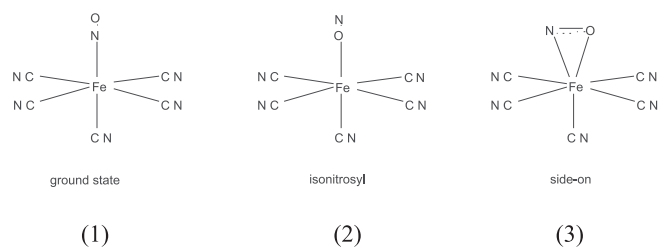


**Figure 1.** Fe-ligand bonding geometry and simplified electronic distribution for ferrous myoglobin complexes of NO, O<sub>2</sub> and CO. Modified from refs. 8 and 13

## LIGAND BINDING AND DISCRIMINATION BY MYOGLOBIN

A wide range of ligands form complexes with either ferrous or ferric Mb in muscle and meat. The small diatomic molecules O<sub>2</sub>, NO and CO all bind reversibly as axial ligand in the sixth position of ferrous Mb, although their affinity for Mb varies significantly despite their similarities regarding size, charge and hydrophobicity.<sup>14-16</sup> Mb complexes with NO or O<sub>2</sub> differ from the complex formed with CO regarding the ability to attain ionic character of the ligand due to variation in dipole moment, and also with respect to iron ligand bonding geometry of the complexes, Figure 1. The complexes

MbFe<sup>II</sup>NO and MbFe<sup>II</sup>O<sub>2</sub> have a bent ligand geometry (112-147°),<sup>17,18</sup> whereas MbFe<sup>II</sup>CO has been found (somewhat depending on the technique applied) to exhibit virtually a linear ligand geometry (155-180°).<sup>19</sup> Several non-heme iron nitrosyl complexes exist and especially nitroprusside, [Fe(CN)<sub>5</sub>NO]<sup>3-</sup>, has been studied as the reactions of nitroprusside mimic the reactivity of metallonitrosyl in physiological environments.<sup>20</sup> The geometry of the ground-state (structure I) has a typical low-spin character with a Fe-NO<sup>+</sup> core and stronger  $\pi$ -back bonding to NO relative to the trans CN<sup>-</sup> resulting in a slight displacement of the iron towards the NO ligand, but still the short Fe-NO bond is linear rather than bent. Following photoexcitation of nitroprusside linkage isomers have been found, e.g. a linear oxygen bound isonitrosyl (structure II) and a side-on complex with NO bound sideways (structure III).<sup>21</sup> Such isomers may also exist in nitrosylated heme protein and alter the reactivity of the heme Fe-NO complex, e.g. during photo-induced degradation of the nitrosylmyoglobin in meat products.



The differences in ligand geometry have been used to explain variation in ligand affinity for Mb, and the observed bond geometries can be assigned either to variations in electrostatic interaction between ligand and amino acid residues or to steric hindrance in the heme pocket. In Table 1, rate constants for ligand association, ligand dissociation and equilibrium constants for binding of various ligands to Mb or heme models are summarized. NO forms the most stable complexes with ferrous Mb, while CO has a significantly lower affinity for Mb closely followed by O<sub>2</sub>. However, studies with site-directed mutants of Mb suggest that the most important factor for overall stability of complexes of Mb is the ability to interact with hydrogen in His64, whereas steric hindrance of this residue is of minor significance.<sup>16</sup> Moreover, the variation in stability observed for MbFe<sup>II</sup>NO and MbFe<sup>II</sup>O<sub>2</sub> can be partly assigned to differences in the rate of bond formation between different ligands and heme iron. NO exhibits practically no energy barrier for bond formation, whereas the bond formation with O<sub>2</sub> involves an energy barrier roughly equal to the energy for ligand escape from the heme pocket.<sup>14</sup>

Not surprisingly, the dynamic of recombination between heme iron and diatomic ligands, e.g. NO, CO, O<sub>2</sub>, depends on the presence or absence of the globin protein.<sup>22</sup> This is further reflected in the differences in the binding constants observed for heme-containing proteins and various modified heme model compounds (see Table 1).

Several studies indicate that coordination of NO to heme iron weakens the fifth coordination position with proximal His93.<sup>23</sup> In addition, lowering of pH will further induce weakening or breaking of the proximal base coordinated to iron. Thus, Duprat et al. have found that when coordination between proximal His93 and heme iron is absent due to low pH or site-directed mutagenesis, the recombination rate of NO and heme iron is very fast resulting in a penta-coordinated state that is 3-fold more stable compared to NO bound in hexa-coordinated Mb.<sup>24,25</sup> This transformation from a hexa- to penta-coordinated NO complex is of biological relevance in heme proteins such as soluble guanylate cyclase,<sup>26,27</sup> and flavo-heme enzymes, like Nitric Oxide Synthases (NOS).<sup>28</sup>

**Table 1.** Rate constants for ligand association ( $k_{\text{ass}}$ ) and ligand dissociation ( $k_{\text{diss}}$ ) together with equilibrium constant ( $K_{\text{eq}}$ ) for ligand binding to ferrous and ferric heme proteins and model compounds

Iron Species	Ligand	$k_{\text{ass}} (\text{M}^{-1} \text{s}^{-1})$	$k_{\text{diss}} (\text{s}^{-1})$	$K_{\text{eq}} (\text{M}^{-1})$	Experimental Conditions	Ref.
FerrousMb	O <sub>2</sub>	$1.4 \times 10^7$	10	$1.4 \times 10^6$	Phosphate, pH 7.0, 20 °C	15
	NO	$1.7 \times 10^7$	$1.2 \times 10^{-4}$	$1.4 \times 10^{11}$	Phosphate, pH 7.0, 20 °C	16
	CO	$5.1 \times 10^5$	0.019	$2.7 \times 10^7$	Phosphate, pH 7.0, 20 °C	15
FerricMb	NO	$1.9 \times 10^5$	13.6	$1.4 \times 10^4$	H <sub>2</sub> O, pH 6.5, 25 °C	22
	HF	$4.7 \times 10^4$	2	$2.4 \times 10^4$	Acetate, pH 4.5, 25 °C	29
	F <sup>-</sup>	$5.6 \times 10^{-2}$	$8.7 \times 10^{-4}$	64	Glycine, pH 11.0, 25 °C	29
	NO <sub>2</sub> <sup>-</sup>	233	5.5	42	Tris, pH 7.4, 25 °C	30
Ferrous (TPPS) <sup>a</sup>	NO	$1.8 \times 10^9$	~0	>10 <sup>9</sup>	H <sub>2</sub> O, pH 6.5, 25 °C	22
Ferric(TPPS) <sup>a</sup>	NO	$5.0 \times 10^5$	$5.0 \times 10^2$	10 <sup>3</sup>	H <sub>2</sub> O, pH 6.5, 25 °C	31

<sup>a</sup>Tetra(4-sulfonatophenyl)porphine

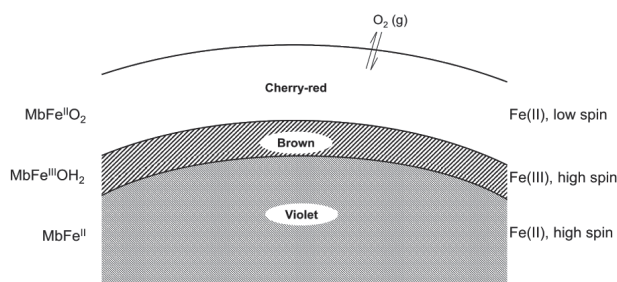
## COLOUR OF FRESH MEAT

The content of Mb in skeletal muscle tissue varies within different animal species, e.g. whale > beef > pork > poultry. Moreover, Mb concentration differs in muscles of the same animal and increases with age, e.g. beef > veal. The fact that whales along with other sea mammals all have relatively high Mb content in their skeletal muscle tissue is supportive of a function of Mb as an oxygen storage protein during prolonged periods of diving. Mb has recently also been isolated from human smooth muscle,<sup>32</sup> and new findings with respect to its role in cardiovascular biology suggest that Mb may also play other roles in the cells.<sup>33</sup>

In freshly cut meat, the primary pigment is an oxygenated form of ferrous Mb, MbFe<sup>II</sup>O<sub>2</sub> (d<sup>6</sup>), while a thin layer of the oxidized form metmyoglobin, MbFe<sup>III</sup>OH<sub>2</sub> (d<sup>5</sup>), will exist at a certain depth in the meat followed by the reduced form deoxymyoglobin, MbFe<sup>II</sup> (d<sup>6</sup>).<sup>9</sup> The localisation of these three forms of Mb in raw meat can be visualised as in Figure 2, and the depth of the MbFe<sup>II</sup>O<sub>2</sub> ( $x$  in mm) will be determined by the partial pressure of oxygen ( $p\text{O}_2$ ) in the air or headspace of a package surrounding the meat surface and may be calculated from Equation (1):

$$x_{\text{MbFe}^{\text{II}}\text{O}_2} = \sqrt{\frac{2 \times C_0 \times D}{A_0}} \quad (1)$$

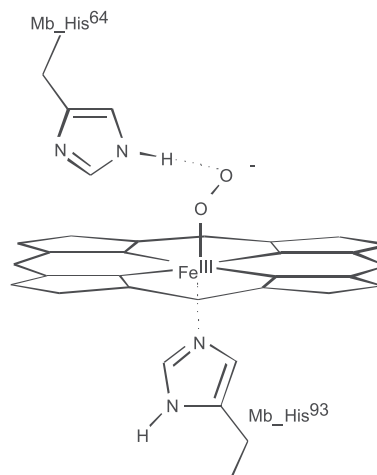
where  $C_0$  is total oxygen concentration as determined by  $p\text{O}_2$ ,  $D$  is the diffusion coefficient of O<sub>2</sub> and  $A_0$  is the O<sub>2</sub> consumption rate due to activity of endogenous enzymes.



**Figure 2.** Schematic illustration of myoglobin forms in layers of fresh meat in equilibrium with atmospheric oxygen at the surface showing ligand coordinated and iron atom spin state

The bond formation between ferrous heme iron and O<sub>2</sub> involves the p-orbital electrons of the O-atom, and as this ligand has strong electron withdrawing ability the resulting complex has partial ionic

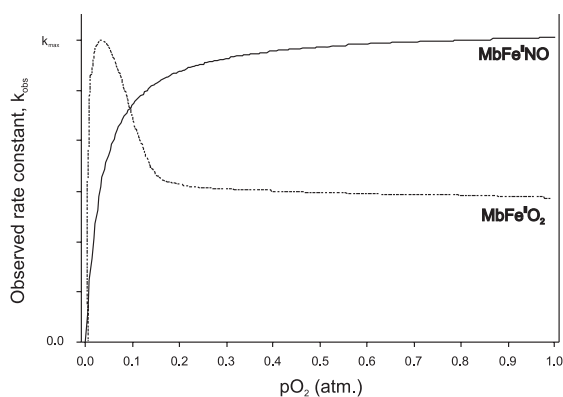
character, often described as a partial ferric heme iron with superoxide anion coordinated.<sup>8</sup> Moreover, this electronic structure of the complex also yields a bent geometry relative to the heme plane and allows the O<sub>2</sub> molecule to interact with an amino acid residue (His64) in the protein back-bone present in the distal heme pocket as shown in Figure 3.



**Figure 3.** Electronic distribution and metal-ligand geometry of Fe-O<sub>2</sub> in oxymyoglobin illustrating the importance of His binding

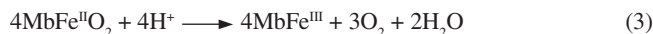
The reaction known as autoxidation of MbFe<sup>II</sup>O<sub>2</sub> in which the red Mb derivative is spontaneously oxidized in the presence of molecular oxygen (eq. 2), is quite complex and has received much attention as this process besides being important for meat quality is also crucial in physiology.<sup>34</sup> Site-directed mutagenesis of Mb amino acid residues in the heme crevice reveals a pivotal role of these residues in determining the rate of autoxidation.<sup>35</sup> Particular the His64 has been found important for the robustness as native MbFe<sup>II</sup>O<sub>2</sub> has an observed rate constant for autoxidation of  $1.5 \times 10^{-5} \text{ s}^{-1}$ , while a mutant in which His64 was replaced by Ala exhibits a rate constant of  $0.016 \text{ s}^{-1}$ . The rate of autoxidation of MbFe<sup>II</sup>O<sub>2</sub> depends on the oxygen pressure initially showing a sharp linear increase with increasing  $p\text{O}_2$ , passing through a maximal rate at a temperature-dependent  $p\text{O}_2$ , and then leading to a significant decrease in the rate as the  $p\text{O}_2$  increases further,<sup>34</sup> as is schematically illustrated in Figure 4. This dependency of the autoxidation rate is consistent with a bimolecular mechanism in which the fraction of MbFe<sup>II</sup> reacts with unbound O<sub>2</sub> by an outer-sphere electron transfer yielding MbFe<sup>III</sup> and O<sub>2</sub><sup>-</sup> as seen in Equation 2.

The logarithmic transformed rate constant for autoxidation of MbFe<sup>II</sup>O<sub>2</sub> depends linearly on pH under acidic conditions in support of



**Figure 4.** Schematic curves of effect of oxygen partial pressure ( $pO_2$ ) on pseudo first order rate constants for autoxidation of  $MbFe^{II}O_2$  and  $MbFe^{II}NO$

specific-acid catalysis with the stoichiometry shown in eq. 3.<sup>36</sup> The detailed mechanism is described by a two-state model with a single protolytic group, namely the distal His64 residue that when protonated forms H-bond with coordinated dioxygen and hereby assists in transferring a proton from the solvent to the bound, polarized dioxygen. A positive kinetic salt effect on the specific acid-catalyzed autoxidation of  $MbFe^{II}O_2$  has been demonstrated, as the rate of proton-assisted nucleophilic replacement of  $O_2^-$  is found to increase with increasing ionic strength.<sup>36</sup> Porcine  $MbFe^{II}O_2$  reacts with a significant lower rate compared to the Mb complex isolated from bovine, corvine and ovine species and with different temperature dependence.<sup>37</sup> For the acid-catalyzed autoxidation of bovine  $MbFe^{II}O_2$  the reported activation enthalpy  $\Delta H^\ddagger = 117 \text{ kJ mol}^{-1}$  and activation entropy  $\Delta S^\ddagger = 172 \text{ J mol}^{-1} \text{ K}^{-1}$  demonstrate a very high activation barrier and a dissociative activation, respectively.<sup>36,37</sup> Accordingly, the initial dissociation of oxygen as shown in Equation 2 seems to be rate-determining rather than the subsequent electron transfer to create the superoxide anion radical. The observed kinetic salt effect for the rate of  $MbFe^{II}O_2$  autoxidation is very similar to that observed for autoreduction of certain hypervalent and prooxidative forms of Mb, as discussed in more detail below.

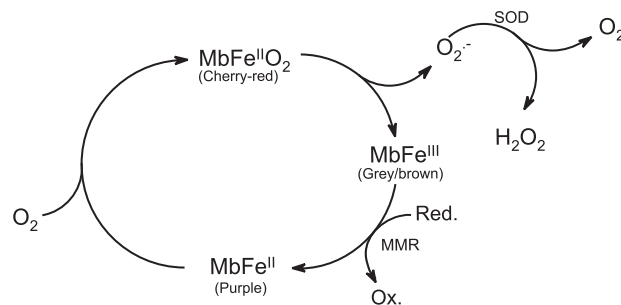


At high hydrostatic pressures, two opposing factors were found to affect the observed rate for autoxidation of  $MbFe^{II}O_2$ : expansion in the transition state (activation volume  $\Delta V^\ddagger$  of  $+12.7 \text{ ml mol}^{-1}$ ), is in agreement with the positive value of  $\Delta S^\ddagger$  that decreases the rate and a pressure-induced decrease in solution pH increases the rate.<sup>38</sup>

The electronic structure of the  $Fe^{II}-O_2$  heme moiety corresponds to a structure with ionic character as shown in Figure 3, in effect resulting in a positive kinetic salt effect at reduced pH as in meat. During the transformation of muscle tissue to meat, post-mortem anaerobic glycolysis forms lactic acid, which helps to prevent microbial spoilage, but also increases autoxidation and decrease the colour stability. From a meat quality viewpoint, the desirable bright red pigment,  $MbFe^{II}O_2$ , should be protected and preserved during storage, which may be achieved using modified atmosphere packaging with gas mixtures having high  $pO_2$  tensions (60-80%) along with  $CO_2$  (20-30%) to inhibit microbial growth.<sup>39</sup> Moreover,  $MbFe^{II}O_2$  is very sensitive to light exposure and photooxidation of  $MbFe^{II}O_2$  is strongly wavelength dependent with quantum yields, as determined using continuous wave photolysis with monochromatic light, having an exponential increase with increasing energy of the irradiation.<sup>40</sup> Hence, UV-light with  $\lambda = 254 \text{ nm}$  was found to give a 4,700-fold increase

in relative rate of photooxidation in comparison to green light ( $\lambda = 546 \text{ nm}$ ). Therefore, the wavelength distribution is critical for colour stability as was shown for minced raw beef during frozen storage for which surface discoloration is often encountered (see Table 2).<sup>41</sup>

In raw meat from freshly slaughtered animals the enzyme system metmyoglobin reductase (MMR) is still active and reduces  $MbFe^{III}$  to the physiological active  $MbFe^{II}$ , which coordinates  $O_2$  and reforms the bright red colour.<sup>42,43</sup> In Figure 5 the so-called colour cycle is shown for fresh meat with various forms of Mb. The colour stability of meat during slaughter, de-boning, storage and retail display depends on post-mortem pH, de-boning/storage temperature,  $O_2$  tension, possible addition of salt, packaging and light exposure.



**Figure 5.** Colour cycle showing interchange of various myoglobin derivatives in fresh meat during storage. The attractive, cherry-red oxymyoglobin ( $MbFe^{II}O_2$ ) can be oxidised in an uncatalysed reaction into grey/brown metmyoglobin ( $MbFe^{III}$ ) and superoxide anion ( $O_2^-$ ), which assisted by the enzyme superoxide dismutase (SOD) dismutates into oxygen and hydrogen peroxide ( $H_2O_2$ ). As long as reducing co-factors are available in raw meat  $MbFe^{III}$  can be reduced to deoxymyoglobin ( $MbFe^{II}$ ) by the enzyme system Metmyoglobin Reductase (MMR). This reduced form readily binds molecular oxygen to yield the oxygenated cherry-red form of the pigment

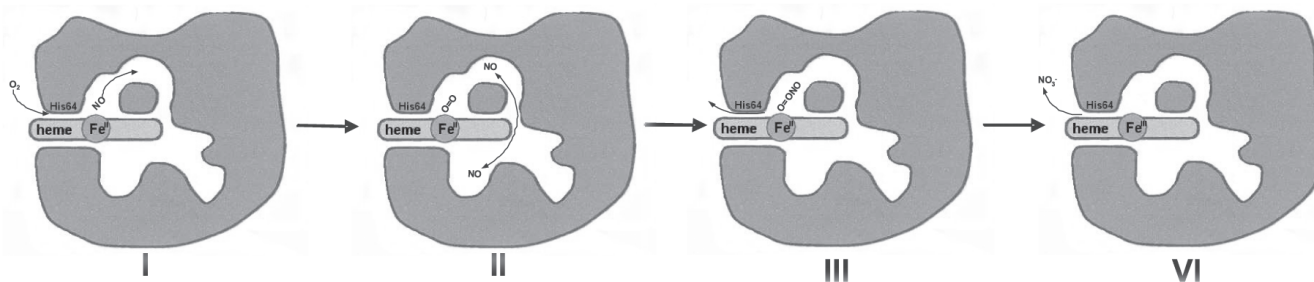
The uncatalyzed autoxidation of  $MbFe^{II}O_2$  yields  $O_2^-$  as initial reaction product, which will dismutate under the influence of superoxide dismutase (SOD) activity or as a result of acid catalysis generating  $H_2O_2$  and  $O_2$ .  $H_2O_2$  is crucial for formation of prooxidative heme species in meat,<sup>44</sup> as will be discussed in more detail below.

## COLOUR OF CURED MEAT PRODUCTS

A general review of the chemistry of Mb and NO has recently been published,<sup>45</sup> in which the complex reactions occurring in cured meat products are discussed. The formation of the dominating heme pigment, nitrosylmyoglobin ( $MbFe^{II}NO$ ), in nitrite-cured meats involves a complex series of reactions between added nitrate/nitrite and either endogenous or added reductants forming NO that readily associates to heme  $Fe^{II}$  in Mb.<sup>46</sup> The exact reaction pathway from nitrite to NO in the meat matrix is not fully established, but nitrite initially oxidizes the fresh meat pigment  $MbFe^{II}O_2$  to  $MbFe^{III}$ , as shown in Equation 4. This reaction is observed as a transient discoloration of the meat surface immediately after the addition of nitrite. The principal nitrosylating agent in cured meat has not been identified yet but several species have been suggested including the acid anhydride of nitrous acid, which, however, is only present in a minute fraction at the normal pH of meat with a value well above the  $pK_a$  of  $HNO_2$ . Nitrosylchloride,  $NOCl$ , which is known to act as a strong nitrosylating agent,<sup>47</sup> is more likely the reactant also in the meat matrix where it may be formed following the simultaneous addition of  $NaCl$  and  $NaNO_2$ .<sup>48</sup>







**Figure 6.** Reaction mechanism suggested for the autoxidation of nitrosylmyoglobin involving two consecutive steps. Schematic drawing of myoglobin molecule was adapted from ref. 54, while the reaction mechanism is depicted according to ref. 53

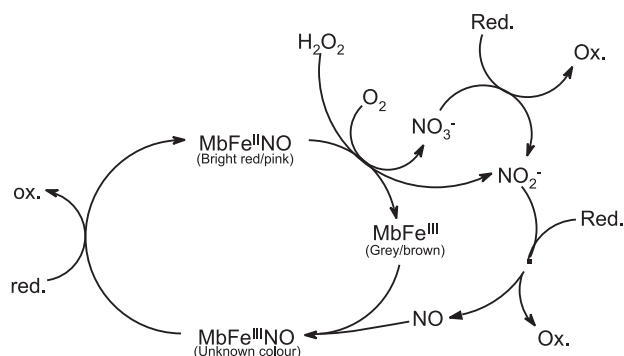
NO is unique as ligand as it binds to both the ferric and ferrous heme iron in Mb with equilibrium constants of  $1.4 \times 10^4 \text{ M}^{-1}$  and  $\geq 10^{11} \text{ M}^{-1}$ , respectively.<sup>22</sup> At higher pH values nitrosylmetmyoglobin ( $\text{MbFe}^{\text{II}}\text{NO}$ ) can via nucleophile attack of  $\text{OH}^-$  undergo reductive nitrosylation yielding  $\text{MbFe}^{\text{II}}\text{NO}$ ,<sup>49</sup> although the significance of this pathway is uncertain in the meat matrix. The meat pigment formed during nitrite-curing has been described as a dinitrosyl complex of myoglobin with a NO coordinated both at the distal and proximal side of the heme.<sup>46:50</sup> The final proof of a pentacoordinate mononitrosyl myoglobin as the principal pigment was presented by Bonnett and co-workers employing electron spin resonance (ESR) spectroscopy.<sup>51</sup>

There is several studies concerned about the chemical reactions of the pigment  $\text{MbFe}^{\text{II}}\text{NO}$  in model systems including oxidative degradation under conditions relevant to cured meat products during storage. In contrast to what was found for  $\text{MbFe}^{\text{II}}\text{O}_2$ , autoxidation of  $\text{MbFe}^{\text{II}}\text{NO}$  is not sensitive to pH and to changes in ionic strength as caused by salt addition. The reaction of  $\text{MbFe}^{\text{II}}\text{NO}$  with molecular oxygen, known as autoxidation, is a relatively slow reaction, and the pseudo-first rate constant is very similar to the first-order rate constant found for dissociation of NO from  $\text{MbFe}^{\text{II}}\text{NO}$ . Accordingly, it was suggested that autoxidation should be described by two consecutive reaction steps,<sup>52</sup> i.e. initial association of  $\text{O}_2$  to coordinated NO forming an intermediate peroxy-nitrite complex followed by an electron transfer and dissociation of the reaction products. More recent results, however, show that the initial step is a ligand exchange between coordinated NO and  $\text{O}_2$  present in solution.<sup>53</sup> Thus, the rate constant of the initial and rate-determining step exhibits a sharp linear increase in the rate, when  $p\text{O}_2$  is increased from low to atmospheric pressure, in which saturation is observed (Figure 4). This indicates the ligand exchange to exhibit saturation behaviour with respect to the incoming  $\text{O}_2$  ligand. The second reaction step has been assigned to a fast bimolecular oxidation of  $\text{MbFe}^{\text{II}}\text{O}_2$  induced by NO ( $k_2 \approx 10^7 \text{ M}^{-1} \text{ s}^{-1}$ ). This reaction is now believed to be important as a clearing pathway for excess NO in mammalian skeletal and cardiac muscle tissue under conditions of nitrosative stress.<sup>54</sup> The NO molecule initially coordinated to  $\text{MbFe}^{\text{II}}\text{NO}$  seems to remain in the vicinity of the heme iron complex of  $\text{O}_2$  formed as intermediate in the exchange reaction. It further seems possible that NO is situated within the protein structure in which several cavities have been identified.<sup>55</sup> It should be noted that NO is not released from the Mb complex into the solvent according to this more detailed mechanism, which was based on additional experiments including bimolecular reactions between  $\text{MbFe}^{\text{II}}\text{NO}$  and  $\text{MbFe}^{\text{II}}\text{O}_2$ . Mb accordingly seems to serve as a chemical reactor for small molecules like  $\text{O}_2$  and NO as is depicted in Figure 6. The more detailed mechanism for autoxidation of  $\text{MbFe}^{\text{II}}\text{NO}$  is accordingly suggested to be initiated by an  $\text{O}_2$  substitution of NO at the heme iron centre (Figure 6.I). NO is subsequently located in one or more protein cavities,<sup>6</sup> as has been identified by Xe-binding (Figure 6.II). NO attacks coordinated  $\text{O}_2$  from

the cavities to form a transient, coordinated  $\text{ONOO}^-$  (Figure 6.III), which subsequently dissociates following isomerisation to nitrate,  $\text{NO}_3^-$ , that is expelled from the heme cleft (Figure 6.IV).

Figure 7 shows the involvement of Mb in the colour cycle of cured meat products, and it includes the possible reformation of the pink form after oxidation to brown  $\text{MbFe}^{\text{III}}$ . Such colour re-establishment has been observed in studies of colour stability of cooked cured ham during storage,<sup>56</sup> although the initial colour intensity was never fully restored.<sup>56:57</sup> The pigment of nitrite-cured meat is much more susceptible to light-induced degradation than the pigment of fresh meat. However, the quantum yield for  $\text{MbFe}^{\text{II}}\text{NO}$  shows only moderate wavelength dependence in contrast to  $\text{MbFe}^{\text{II}}\text{O}_2$ , but the quantum yields are several magnitudes higher for  $\text{MbFe}^{\text{II}}\text{NO}$  compared to  $\text{MbFe}^{\text{II}}\text{O}_2$  for wavelength of relevance to food storage (Table 2).<sup>40:58</sup> Furthermore, the quantum yield for photooxidation of  $\text{MbFe}^{\text{II}}\text{NO}$  increases linearly with  $p\text{O}_2$ ,<sup>58</sup> even at very low oxygen tension as demonstrated for the pressure interval  $0.0010 < p\text{O}_2 < 0.0150$  atmospheres.<sup>59</sup> Interestingly, a comparison of the stoichiometries for thermal and photo-induced oxidation of  $\text{MbFe}^{\text{II}}\text{NO}$  shows as expected a 1:1 stoichiometry for  $\text{O}_2$  and  $\text{MbFe}^{\text{II}}\text{NO}$  during thermal oxidation, while photooxidation results in higher pigment degradation relative to the  $\text{O}_2$  present. This corresponds to a stoichiometry with approximately 20% excess degradation of  $\text{MbFe}^{\text{II}}\text{NO}$  relative to the  $\text{O}_2$  present, probably due to involvement of radical intermediates and chain reactions.

Evidence of damage to apomyoglobin (apoMb) during thermal



**Figure 7.** Colour cycle of myoglobin derivatives in cured meat during storage. The attractive pink nitrosylmyoglobin ( $\text{MbFe}^{\text{II}}\text{NO}$ ) can be oxidised both by  $\text{H}_2\text{O}_2$  or by molecular oxygen (autoxidation) to yield grey/brown metmyoglobin ( $\text{MbFe}^{\text{III}}$ ) and nitrite or nitrate, respectively. In meat added reducing agents the pools of nitrate/nitrite can be reduced back to NO, which binds to metmyoglobin yielding the complex nitrosylmetmyoglobin ( $\text{MbFe}^{\text{II}}\text{NO}$ ). This complex can undergo reduction, thereby returning to  $\text{MbFe}^{\text{II}}\text{NO}$ . During storage of nitrite-cured meats such discoloration followed by reformation of pink pigment is directly observable

**Table 2.** Quantum yields and relative rates for photooxidation of oxymyoglobin and nitrosylmyoglobin exposed to monochromatic irradiation at 15 °C

$\lambda_{\text{irr}}$ (nm)	MbFe <sup>II</sup> O <sub>2</sub> <sup>a</sup>		MbFe <sup>II</sup> NO <sup>b</sup>	
	$\Phi_{\text{irr}}$ (mol einstein <sup>-1</sup> )	Relative rate	$\Phi_{\text{irr}}$ (mol einstein <sup>-1</sup> )	Relative rate
254	$1.6 \times 10^{-2}$	4700	$6.9 \times 10^{-3}$	41
313	$7.9 \times 10^{-4}$	150	$2.3 \times 10^{-3}$	7
334	$1.8 \times 10^{-4}$	50	$1.6 \times 10^{-3}$	6
366	$3.9 \times 10^{-5}$	10	$1.5 \times 10^{-3}$	7
405	$1.2 \times 10^{-5}$	12	$1.1 \times 10^{-3}$	14
436	$7.0 \times 10^{-6}$	3	$8.6 \times 10^{-4}$	7
546	$7.0 \times 10^{-6}$	1	$4.3 \times 10^{-4}$	1

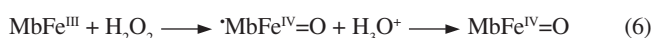
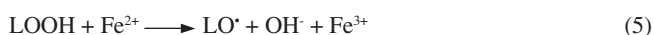
<sup>a</sup>Results from ref. 41; <sup>b</sup>results from ref. 59

oxidation of MbFe<sup>II</sup>NO has also been presented as repeated oxidation and nitrosylation of MbFe<sup>II</sup>NO after several cycles has been found to yield hemichrome, a denatured ferric Mb form with two His residues bound to the central iron atom,<sup>60</sup> which is detectable by ESR spectroscopy.

### MYOGLOBIN AND OXIDATIVE PROCESSES IN BIOLOGICAL SYSTEMS

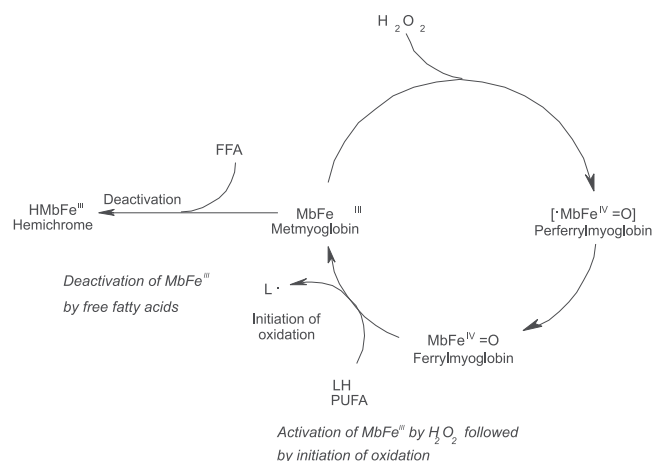
The redox-activities involved in the discoloration of both fresh meat and nitrite-cured meat products affect the oxidative stability of the lipid fraction of the product, as iron redox cycling seems to initiate peroxidation and formation of low-molecular weight compounds responsible for off-flavours and rancidity. The interaction between meat discoloration and lipid oxidation in meat involves the brown MbFe<sup>III</sup>, which has been found to have pseudo-peroxidase activity and which forms several prooxidative Mb species during the catalytic cycle following reaction with H<sub>2</sub>O<sub>2</sub> and other peroxides. MbFe<sup>III</sup> also takes part in the propagation of lipid peroxidation by cleavage of lipid hydroperoxides.<sup>44</sup> H<sub>2</sub>O<sub>2</sub> is central in triggering of processes leading to oxidative rancidity in fresh meat, as O<sub>2</sub><sup>•-</sup> formed during autoxidation of MbFe<sup>II</sup>O<sub>2</sub> yields H<sub>2</sub>O<sub>2</sub> either spontaneously or mediated by SOD. In addition, H<sub>2</sub>O<sub>2</sub> may also be produced in significant quantities as a result of growth of the catalase-negative lactic acid bacteria on the meat surface.

Transition metal redox couples such as Fe<sup>III</sup>/Fe<sup>II</sup> or Cu<sup>I</sup>/Cu<sup>I</sup> have standard reduction potentials allowing catalytic decomposition of lipid hydroperoxides (LOOH) to form LO<sup>•</sup> radicals (Equation 5), and can thereby initiate the chain reaction characteristic for lipid autoxidation. Heme proteins or free heme groups released from proteins also have the ability to enhance lipid peroxidation processes,<sup>60</sup> and Mb has been active in this respect especially under acidic conditions. Mb reacts with H<sub>2</sub>O<sub>2</sub> in a two-electron process in which a hypervalent perferrylmyoglobin, 'MbFe<sup>IV</sup>=O, is formed with a tyrosyl radical at Tyr103 (Equation 6). The initially formed hypervalent Mb then undergoes the so-called autoreduction to form ferrylmyoglobin, MbFe<sup>IV</sup>=O.<sup>61</sup> Both of these hypervalent Mb species are known to initiate lipid peroxidation by hydrogen abstraction from fatty acids.<sup>62</sup> However, it should be noted that free heme per se has a higher pseudo-peroxidase activity compared to protein bound heme.<sup>63</sup>



Not surprisingly, it has been shown that phenolic acids, known as

efficient antioxidants, inhibit the MbFe<sup>III</sup>/H<sub>2</sub>O<sub>2</sub> induced oxidation of low-density lipoproteins.<sup>64</sup> More intriguing is the finding that free fatty acids seem to prevent formation of activated hypervalent forms of Mb<sup>65</sup> and hemoglobin (Hb)<sup>66</sup> favouring formation of the inactive hemichromes, as shown in Figure 8 for Mb. This inhibitory effect of free fatty acids on the formation of hypervalent Mb species has been investigated for conditions of excess of saturated fatty acids. Stearic acid and palmitic acid were both found to reduce 'MbFe<sup>IV</sup>=O formation from MbFe<sup>III</sup> and H<sub>2</sub>O<sub>2</sub>, while similar quantities of the monounsaturated oleic acid did not affect the 'MbFe<sup>IV</sup>=O formation.<sup>67</sup> However, it is not known whether this antioxidative mechanism is important in biological systems or in muscle-based food products, but free fatty acids have been found to increase in concentration during ischemia reperfusion,<sup>68</sup> and extensive lipolysis also occurs during long maturation of dry-cured meat products, which attain an exceptional good oxidative stability.<sup>69,70</sup> In this context, it should be noted that C<sub>8</sub> – C<sub>14</sub> saturated fatty acids recently have been found to inhibit the activity of cyclooxygenase enzymes, which are responsible for initiating inflammation processes in the body.<sup>71</sup>



FFA = free fatty acids; PUFA = polyunsaturated fatty acids

**Figure 8.** Prooxidative cycle of Mb activated by H<sub>2</sub>O<sub>2</sub> and proposed mechanism for inactivation by free fatty acids forming hemichrome

The rate of MbFe<sup>II</sup>O<sub>2</sub> autoxidation increases at reduced oxygen tension,<sup>36,37</sup> which again increases the oxidative stress of membrane lipids, as O<sub>2</sub><sup>•-</sup> is a product of MbFe<sup>II</sup>O<sub>2</sub> autoxidation. The effect on lipid oxidation of the post-mortem pH decrease in muscle-based foods<sup>60</sup> has a parallel in the acidification and reduced oxygen tension occurring in tissue exposed to ischemia reperfusion during surgery,<sup>72</sup> which also increases autoxidation of oxygenated heme proteins. However, the rate for autoreduction of the prooxidative species, MbFe<sup>IV</sup>=O, has also been found to be subject to specific acid catalysis and is accordingly pH dependent with a modest temperature dependence corresponding to the activation parameters:  $\Delta H^\ddagger = 58.5 \text{ kJ mol}^{-1}$ ,  $\Delta S^\ddagger = 2.7 \text{ J mol}^{-1} \text{ K}^{-1}$ .<sup>73</sup> A positive kinetic salt effect was also observed for the autoreduction of MbFe<sup>IV</sup>=O to yield MbFe<sup>III</sup>, and any MbFe<sup>IV</sup>=O formed in either fresh meat or cured meat products will be deactivated due to the low pH conditions and the presence of salt in competition with oxidation of the membrane phospholipids.

The effects of glycation of proteins in relation to various physiological dysfunctions is an emerging field within the medical sciences, and the covalent binding of blood glucose to circulating Hb is involved in several diabetes related pathological conditions.<sup>74-76</sup> Glycation of Mb was recently demonstrated to enhance the prooxidative activity of Mb and to increase release of free iron ion

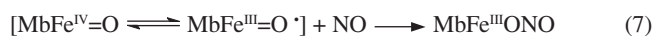
from this heme protein.<sup>77</sup> Similar effects should be studied for meat products with sugar added as flavouring agent or as substrate for microbial starter cultures.

## HEME PROTEINS AND NO<sup>•</sup> IN OXIDATIVE PROCESSES

Once initiated, lipid peroxidation, a chain reaction, will be further enhanced by radicals or redox active metals or will be inhibited by free radical scavenging compounds. Both heme proteins and NO belong to a class of chemical compounds that can be active as promoters or inhibitors of lipid peroxidation,<sup>78</sup> and their role in lipid peroxidation will be discussed with special focus on the reactivity of NO towards reaction intermediate in lipid oxidation.

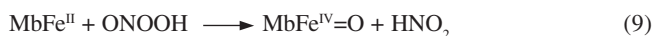
NO can act as a prooxidant or an antioxidant depending on the concentration of NO and the absence or presence of O<sub>2</sub><sup>•-</sup>.<sup>79</sup> NO reacts extremely fast with O<sub>2</sub><sup>•-</sup> (k<sub>2</sub> = 10<sup>9</sup> M<sup>-1</sup> s<sup>-1</sup>) and the reaction product is the strong prooxidant peroxynitrite, ONOO<sup>-</sup>.<sup>80</sup> Equimolar fluxes of O<sub>2</sub><sup>•-</sup>, H<sub>2</sub>O<sub>2</sub> and NO have been found to enhance the oxidation of a substrate (dihydrohodamine) in a model system containing MbFe<sup>III</sup>, while further increases in NO concentration significantly reduced oxidation via inhibition of <sup>•</sup>MbFe<sup>IV</sup>=O/MbFe<sup>IV</sup>=O formation.<sup>81</sup> Another study showed that NO protects cardiomyocytes against *tert*-butyl hydroperoxide-induced formation of non-protein and protein-centered free radical species and concomitant peroxidation of membrane phospholipids, and NO was concluded to be an important antioxidant in heart tissue.<sup>82</sup> In contrast, NO formation during ischemia reperfusion of hearts isolated from wild-type or NOS knock-out mice showed a contribution of NO to oxidative injury, most likely due to interaction with O<sub>2</sub><sup>•-</sup>.<sup>83</sup> Recent findings with transgenic mice with an over-expression of NOS, which lack Mb in their cardiac tissue, indicate that excessive formation of NO leads to heart insufficiencies, thus suggesting that Mb under normal circumstances can be an important factor in protecting the heart from nitrosative stress.<sup>84</sup> Thus, in light of these conflicting results, the role of NO in heart tissue under oxidative stress still remains to be clarified.

NO and its interaction with heme iron/Mb has been suggested to prevent oxidative rancidity in nitrite-cured meat.<sup>85;86</sup> Oxidative rancidity is seldom encountered in nitrite-cured meat due to the antioxidative capacity of MbFe<sup>II</sup>NO and possibly also other nitrite-derived compounds.<sup>46</sup> So far, only few studies have investigated the isolated effect of MbFe<sup>II</sup>NO in oxidative processes, but in a carotene-linoleate model system, it has been found that 2-10 μM of MbFe<sup>III</sup> or MbFe<sup>II</sup>O<sub>2</sub> acted as a prooxidative species. In contrast, MbFe<sup>II</sup>NO at all investigated concentrations did not act as a prooxidant, and MbFe<sup>II</sup>NO even inhibits the prooxidative effect of 2 μM MbFe<sup>III</sup>.<sup>87</sup> MbFe<sup>II</sup>NO, in presence of excess MbFe<sup>III</sup>, has further been found to significantly inhibit oxygen consumption in a lipid peroxidating model system with methyl linoleate as substrate.<sup>88</sup> NO has been found to inactivate the highly oxidizing ferryl Mb species involved in oxidative stress.<sup>89</sup> The reaction mechanism includes two steps with a rapid initial formation of a reaction intermediate, nitritometmyoglobin (MbFe<sup>III</sup>ONO), which subsequently decays on a longer time scale (Equations 7-8). The second order rate constant for MbFe<sup>IV</sup>=O and NO is found to be 1.8 × 10<sup>7</sup> M<sup>-1</sup> s<sup>-1</sup> at pH 7.5 and 20°C, while the first order rate constant for decay of the reaction intermediate is 3.4 s<sup>-1</sup>. Thus, it seems that free NO in solution may either inhibit or in the presence of O<sub>2</sub><sup>•-</sup> promote lipid peroxidation. However, when NO is bound to Mb, it may act as a bioactive reservoir that can be released to scavenge OH<sup>•</sup> or lipid derived radicals.



The ability of nitrite (the oxidation product of NO) to inactivate MbFe<sup>IV</sup>=O has been found to occur at a markedly lower rate with a second order rate constant between 13-16 M<sup>-1</sup> s<sup>-1</sup> determined at ambient temperatures.<sup>89;90</sup> Activation parameters of ΔH<sup>‡</sup> = 30 kJ mol<sup>-1</sup> and ΔS<sup>‡</sup> = -123 J mol<sup>-1</sup> K<sup>-1</sup> are indicative of an associative activation with a rate-determining intra-molecular electron transfer to yield a cation radical <sup>•</sup>MbFe<sup>III</sup>-O<sup>•</sup>.

The strong prooxidant ONOO<sup>-</sup> has been found to cause rapid conversion of MbFe<sup>II</sup>O<sub>2</sub> to MbFe<sup>III</sup> under the conditions expected in muscle foods, and the presence of CO<sub>2</sub> and lowering of pH seem to reduce MbFe<sup>II</sup>O<sub>2</sub> degradation slightly.<sup>91</sup> In the reaction between MbFe<sup>II</sup>O<sub>2</sub> and ONOO<sup>-</sup>, it has been found that ONOO<sup>-</sup> oxidizes the small fraction of deoxygenated Mb (in equilibrium with MbFe<sup>II</sup>O<sub>2</sub>) to hypervalent ferryl Mb (Equation 9), while a subsequent reduction by ONOO<sup>-</sup> yields MbFe<sup>III</sup> and peroxynitrite radical (eq. 10).<sup>92</sup> The second order rate constants (20°C and pH 7.3) are found to be very similar and to have values 5.4 × 10<sup>4</sup> and 2.2 × 10<sup>4</sup> M<sup>-1</sup> s<sup>-1</sup>, respectively.



The effect of CO<sub>2</sub> is interesting, since in the presence of 1.2 mM CO<sub>2</sub> the rate of the initial reactions step involving MbFe<sup>II</sup> and ONOO<sup>-</sup> (eq. 9) increases significantly (4.1 × 10<sup>5</sup> M<sup>-1</sup> s<sup>-1</sup>), while the rate of the second reaction step remains practically unaltered.<sup>93</sup> The possible formation of traces of ONOO<sup>-</sup> during the autoxidation of MbFe<sup>II</sup>NO should also be considered, and a recent study of this autoxidation reaction demonstrates the initial rate-determining reaction step to be unaffected by varying levels of CO<sub>2</sub>, while the second reaction step was affected by elevated levels of CO<sub>2</sub>.<sup>94</sup>

Analysis of nitrated amino acid residues in Mb or haemoglobin (Hb) following exposure to variable amounts of ONOO<sup>-</sup> shows that only low quantities of 3-nitrotyrosine can be detected after the reaction with the intact heme proteins.<sup>95</sup> However, when apoMb or a cyano complex of ferric Mb are submitted to similar treatment, significantly larger yields for 3-nitrotyrosine and even lower quantities of nitrated tryptophan is observed in apoMb indicating that the Mb heme iron may act as an efficient scavenger of ONOO<sup>-</sup>, thereby protecting not only its globin part, but also other proteins such as the cytochromes from nitration. This further supports the theory of Mb as a protector of cellular respiration in addition to the function of Mb in facilitated diffusion of O<sub>2</sub> in muscle tissue. Likewise, in a study of the reaction between Hb and ONOO<sup>-</sup> nitrated amino acid residues were not detected, unless large excess of oxidant compounds was added.<sup>96</sup> In contrast, when Hb was incubated with nitrite and H<sub>2</sub>O<sub>2</sub>, nitration of other proteins and apoHb were observed,<sup>97</sup> and a similar reaction pattern has also been observed for Mb.<sup>98</sup> However, blocking of the central iron atom via formation of a cyano Hb complex again showed that it is the pseudo-peroxidase activity of Hb and other heme proteins that accounts for this nitration mechanism.

The mechanism of the early processes involved in the formation of back-bone protein radicals may also have negative effects on the oxidative status of protein and lipids in meats during maturation and storage. In fact, the above-mentioned findings may have implications for meat products packaged in modified atmosphere (MA) where CO<sub>2</sub> is often used to inhibit microbial growth. So far, these mechanisms are quite speculative, as studies have been conducted only in model systems, and their importance in meats or meat products should be investigated in order to fully clarify the role of reactive nitrogen species in oxidative processes taking place for the post-mortem conditions found in muscle foods.

NO can be formed in fresh meat post-slaughter (<8 h) as a



result of remaining activity of NOS in the muscle tissue,<sup>99</sup> and the concomitant formation of O<sub>2</sub><sup>•-</sup> may generate ONOO<sup>•</sup>, which will initiate oxidative processes degrading both lipids and pigments of fresh meat during the initial handling and storage.<sup>91:100</sup> However, further studies are needed to fully assess the potential damaging effect of such reactions, also since hypervalent Mb species so far have not been detected in muscle tissue intended for consumption.

Protein oxidation receives increasing attention in relation to oxidative stress in biological system as it seems linked to certain diseases. For meat and meat products modifications of functional properties due to radical damage of proteins also need to be considered. For model systems containing the free amino acid tyrosine, it has been shown that MbFe<sup>IV</sup>=O accelerates the formation of dityrosine possibly via formation of tyrosyl radicals followed by dimerization.<sup>101</sup> Likewise, the strong prooxidant, ONOO<sup>•</sup>, has been shown to modify specific side chains of amino acid residues in Hb with long-lived tyrosyl radicals being formed upon exposure of erythrocytes to ONOO<sup>•</sup>.<sup>102</sup> In addition, the Cys110 amino acid residue unique to human Mb has been found to form an initial thyl radical upon reaction with ONOO<sup>•</sup> as shown by ESR spin trapping. The final product is, however, either found to be a Mb dimer formed as Tyr103 radicals subsequently form an intermolecular crosslink to another Tyr103 or a 3-nitrotyrosine also in the 103-position.<sup>103</sup>

## CONCLUSION

Colour is an important quality parameter for both fresh meat and nitrite-cured meat products and depends on the redox status and ligand bound to heme iron in Mb. A basic understanding of Mb chemistry is accordingly crucial, and the “inorganic chemistry” of meat should include a quantitative description of Mb complex formation with small ligands, such as O<sub>2</sub>, NO, H<sub>2</sub>O and CO, and the kinetics of transformations of these complexes under varying conditions of temperature, oxygen pressure, pH, ionic strength and light exposure.

The practical aspect of colour stability of meat and meat products for the meat industry and retail trade has initiated numerous investigations over the last 50 years and an increased understanding of the complex chemistry. The discovery of the physiological importance of NO and the possible role of hypervalent Mb and Hb during oxidative stress have added new perspectives to the dynamic description of electron transfer and ligand exchange reactions of these heme pigments. Other functions of Mb than oxygen transport and storage seem to be important and these *in vivo* functions include activity as a pseudo-enzyme in specific muscle tissue similar to NO dioxygenases known from microorganisms. In effect, Mb acts as a cellular protector against nitrosative stress during excess production of NO. Future research should focus on the role of Mb as a mediator of reactions between small molecules important as bio-regulators and include investigations of reaction dynamics possibly occurring within the protein structure using time-resolved spectroscopy.

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