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Review

Heme-iron in lipid oxidation

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Contents

Abstract

Oxygen transport, oxygen storage and oxygen activation in aerobic organisms depend on the iron porphyrin moiety in heme proteins. Under fluctuating oxygen supply and pH decrease the heme pigments like myoglobin and hemoglobin become catalytic in lipid peroxidation by mechanisms different from mechanisms for lipid oxidation by the non-heme-iron lipoxygenase, which is pivotal in energy metabolism. Simpler iron species originating from degradation of heme proteins and other sources bind to negatively charged phospholipids in membranes and catalyze the cleavage of preformed lipid hydroperoxides. Heme initiated lipid peroxidation, involved in pathogenesis in humans, is autocatalytic and forms lipid hydroperoxides and is further linked to cross-linking of proteins. It is also important for quality deterioration of muscle-based food. Heme–iron catalyzed lipid peroxidation is classified into four groups: (i) Fenton-like mechanism; (ii) iron(III)/iron(IV) mechanism; (iii) pseudoperoxidase mechanism; and (iv) iron(II)/iron(IV) mechanism. Partly proteolysed myoglobin becomes catalytic by a Fenton-type one-electron Fe(II)/Fe(III) cycling mechanism, rather than by a pseudoperoxidase mechanism as known from proteolysed cytochromes. Besides hydroxyl-, alkoxyl-, and peroxyl-radicals, nitric oxide is also important in lipid oxidation with a possible role of myoglobin as a nitric oxide dependent antioxidant.

Keywords: Iron catalysis; Myoglobin; Hemoglobin; Lipid hydroperoxides; Oxygen activation; Radical mechanism

1. Introduction

Oxidation of unsaturated lipids depends on oxygen activation and three different paths are recognized as presented

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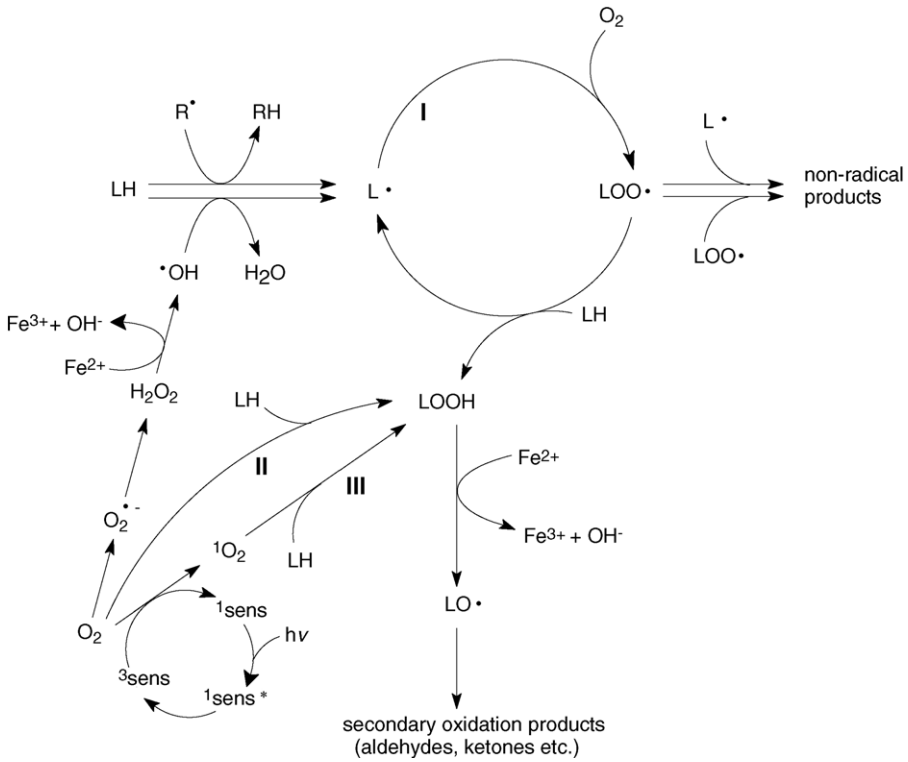

Fig. 1. Lipid oxidation depends on three major reaction paths. (I) Free radical chain reaction initiated by oxygen activation to yield hydroxyl radicals or by radical formation by irradiation or by oxidants. (II) Enzymatic formation of lipid hydroperoxides through lipoxygenase activity. (III) Photosensitized formation of lipid hydroperoxides through formation of singlet oxygen or through direct radical generation.

in Fig. 1. Path I is the so-called "autoxidation" or thermal lipid oxidation by atmospheric air with free radical intermediates, which for oils and fats involves catalysis by traces of transition metal compounds, and as for biological fluids like milk and blood, involves enzymes like the molybdenum containing xanthine oxidase, copper-zinc containing superoxide dismutase, and the iron containing peroxidases as shown in Fig. 2 [1]. Lipoxygenases (path II, Fig. 1) are non-heme-iron enzymes catalyzing dioxygenation of polyunsaturated fatty acids and are widespread in the animal and plant kingdom [2]. Photooxidation involves a photosensitizer like riboflavin or chlorophyll for formation of singlet-oxygen as shown in Fig. 1 as path III, or for direct formation of radicals [3].

Meat is a *post mortem* system and different from living tissue, since the metabolism gradually changes when muscles are no longer continuously supplied with oxygen.

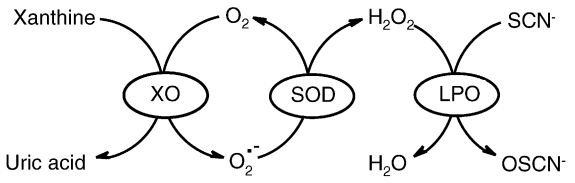

Fig. 2. Enzymatic oxygen activation in milk depends on three transition metal containing enzymes to form the bacteriostatic $OSCN^-$. XO: xanthine oxidase, SOD: superoxide dismutase, LPO: lactoperoxidase (modified from [1]).

A consequence of a change from oxidative to glycolytic metabolism is accumulation of lactic acid, which leads to a drop in pH from 7.4 to approximately 6.0–5.5. Furthermore, the compartmentalisation of intact muscle is more or less destroyed during the post mortem process, which includes protein denaturation and proteolysis, and potential reactants are mixed and non-physiological reactions are becoming possible.

Especially polyunsaturated lipids are labile with respect to oxidation, and the fact that lipid oxidation propagates as a radical chain reaction makes lipids very sensitive to catalysis by traces of metals or small concentrations of modified metalloproteins (see Fig. 1). Lipid hydroperoxides, the primary oxidation products, are further cleaved oxidatively to yield peroxyl radicals or reductively to yield alkoxyl radicals by metals like iron. Both alkoxyl and peroxyl radicals may initiate new reaction chains, while alkoxyl radicals may further cleave to yield aldehydes and other secondary lipid oxidation products. The aldehydes will subsequently modify proteins through formation of Schiff's bases and for unsaturated aldehydes also through crosslinking.

Iron catalysis is of particular interest in relation to thermal lipid oxidation, since iron compounds in aerobic organisms are involved in oxygen transport, oxygen storage, and oxygen activation in the form of heme pigments [4]. Heme pigments, including myoglobin and hemoglobin, may thus under conditions of depletion of oxygen, pH decrease,

or abruptly fluctuating oxygen supply in tissues, as under ischemic conditions, become catalytic.

During the transformation of muscles to meat, similar changes with oxygen depletion and pH decrease occur, and the catalytic properties of myoglobin are enhanced and also affected by proteolysis [5]. On the basis of current results related to the involvement of myoglobin in the oxidative deterioration of meat, we will attempt to classify the different mechanisms by which heme pigments are involved in lipid oxidation in meat; a classification which also may be of interest for other biological systems in which myoglobin and hemoglobin behave prooxidatively. Recently, the physiological role of myoglobin as mainly an oxygen storage molecule has been questioned, since it was found that mice without myoglobin are viable and even fertile [6]. Myoglobin may rather be classified as a biochemical reactor that catalyses reactions among small molecules like O_2, NO, HNO and H_2O_2 [7,8], and which is involved in free radical processes in aerobic organisms. Heme-iron catalysis potentially involves both one- and two-electron transfer processes and the catalytic mechanisms are different from the catalytic mechanism by which lipoxygenases catalyses oxidation of lipids with the different coordination environment for iron as shown in Fig. 3. Lipids are, besides energy storage in both plants and animals, integral parts of cellular structures, where the highly unsaturated and vulnerable phospholipids constitute the bilayers. Heme pigments may under some conditions leak iron to form more simple iron species, which may bind to the negatively charged phospholipids like lecithin and catalyze Fenton-type reactions in the membranes [9,10], as shown in Fig. 4. Under such conditions the membrane may act as a "ligand" for mobile iron(II) and iron(III), and iron is in the Taube classification transferred from inert complexes to labile complexes [11].

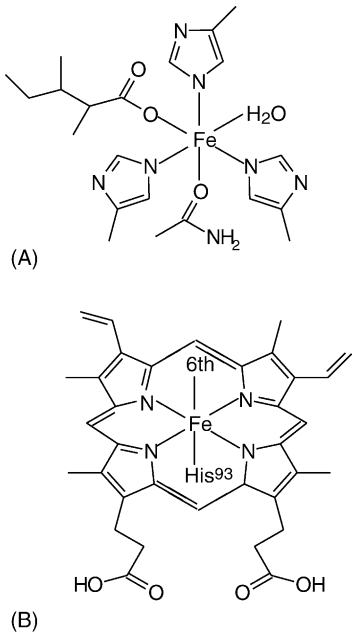

Fig. 3. Iron coordination in heme pigments and lipoxygenases. In contrast to the carboxamid/carboxylate/histidine/water coordination in soybean lipoxygenase (A), the phorphyrin coordination in heme pigments (B) also stabilises higher oxidation numbers than iron(II) and iron(III).

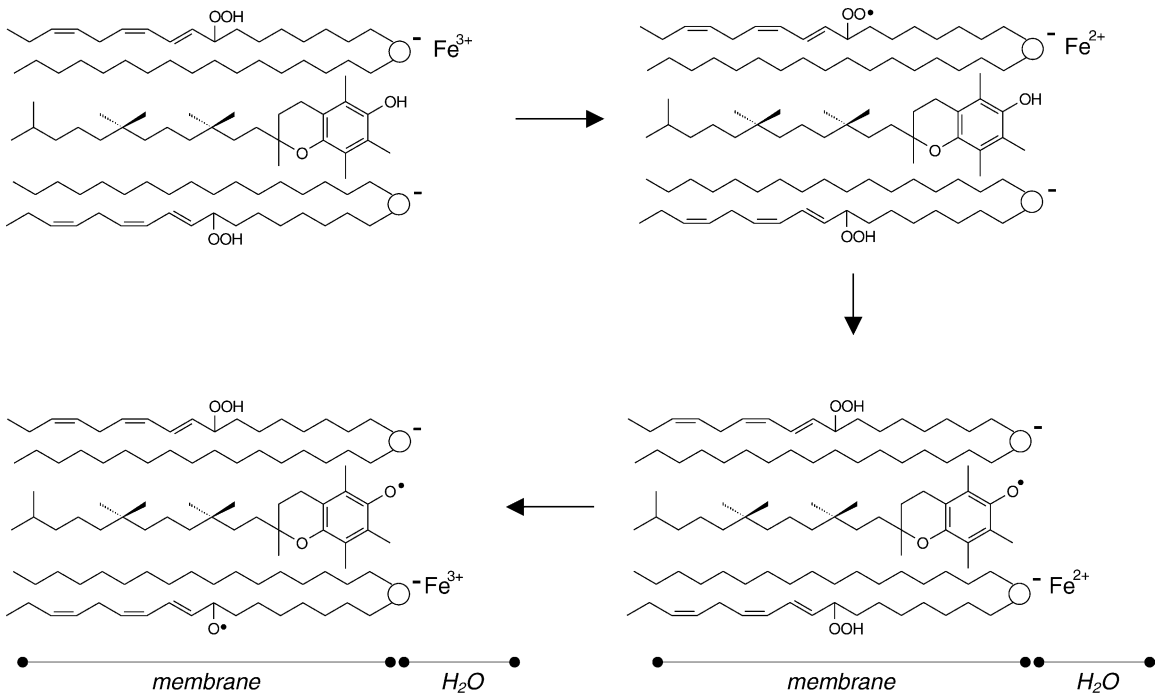

Fig. 4. The negatively charged "heads" of phospholipids like lechitin in bilayer attracts Fe^{3+} and Fe^{2+}, which cleaves preformed lipid hydroperoxides in the membrane oxidatively or reductively, respectively, to yield a Fe(III)/Fe(II) catalytic cycle. α-Tocopherol also shown may regenerate the lipid hydroperoxides.

2. Prooxidative iron species

The ability of iron to alternate between oxidation states makes it reactive in oxidation reactions where electrons are transferred to or from organic compounds, which for one-electron transfer induces radical processes. In acidic aqueous solution, iron in its simplest form is found as $Fe(H_2O)_6^{2+}$ and $Fe(H_2O)_6^{3+}$, each coordinating six water molecules. Most salts of iron(II) are fairly soluble in acidic and neutral solution, but $Fe(H_2O)_6^{2+}$ is readily oxidized by O_2 to $Fe(H_2O)_6^{3+}$, which will deprotonise ($pK_{a1} = 3.0$ [12]) and form soluble hydroxy complexes or precipitate as hydroxy-polymers [13,14]. Hence, the most stable form of iron at physiological oxygen concentrations is complexes of Fe^{3+}, as found, e.g., in the storage protein ferritin. A variety of ligands can, however, replace the H_2O-molecules, and thereby the solubility and redox equilibrium of iron is altered [13,15,16], forming various iron species (including the heme proteins), which through their redox activity influence oxidation processes in biological systems [15,17].

The chemistry of iron species is closely linked to oxygen chemistry in aerobic organisms, as molecular oxygen is found as a ligand in iron biomolecules. Thus, iron may serve as a shuttle between oxygen and biomolecules and allow electron transfers, which are otherwise impossible due to spin restrictions [16,18]. As an example, heme proteins (the cytochromes) catalyze the stepwise reduction of oxygen to water through the so-called electron transport chain, where electron transfer is mediated by shifts between oxidation state +2 and +3 (and possibly +4) of heme-iron [15,17].

In vivo, a close regulation of iron metabolism is nature's attempt to prevent reactive iron species in participating in uncontrolled oxidation reactions. Nevertheless, there is strong evidence that iron-mediated oxidation occurs in vivo [17,19], and the significance and mechanism of reactions with low molecular iron species and heme-iron species are being extensively studied. Both heme-iron species and simpler iron species have been identified as oxidation catalysts in muscle tissue (see, e.g., the review [4]). A final establishment of the nature of the prooxidative iron species in tissue has not been reached; although an overwhelming number of studies have dealt with in vivo oxidation. The simpler iron species may, however, also be activated through cycling with higher oxidation numbers, so-called ferryl compounds, as has been studied in detail by Kremer [20].

Oxidation of organic compounds in the presence of iron and hydrogen peroxide is usually termed Fenton chemistry [21], to honour the classical studies on oxidation of tartaric acid performed by H.J.H. Fenton before year 1900 [22]. The reactive oxygen species, the hydroxyl radical ($^{\bullet}OH$), the superoxide anion ($O_2^{\bullet -}$) and its conjugate acid $HO_2^{\bullet}$ are generally proposed to be involved in Fenton chemistry, and even though a finite mechanism of the reaction between iron and hydrogen peroxide is still not established [21], the Fenton reaction is widely referred to as Eq. (1), and is regarded as a common source of hydroxyl radicals.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + OH^{-} \quad (1)$$

The reduction potential of $^{\bullet}OH$ is 2.3 V [23], and the high reactivity of $^{\bullet}OH$ fairly explains the ability of mixtures of iron species and H_2O_2 to initiate oxidation in various systems. It has been proposed that the reactive product of the reaction of Eq. (1) is not a hydroxyl radical, but an iron–oxo (FeO^{2+}) or ferryl species (Fe(IV)=O), with iron in the oxidation state +4 [20,21,24]. A two-electron transfer and not a one-electron transfer thus results from the reaction between Fe^{2+} and H_2O_2:

$$Fe^{2+} + H_2O_2 \rightarrow FeO^{2+} + H_2O \quad (2)$$

For some heme-iron species, involvement of the ferryl state of iron (i.e. a +4 state) in oxidation reactions is well established. However, a further consideration of whether ferryl iron species in general (and not $^{\bullet}OH$) are the reactive products of iron–hydrogen peroxide reactions has been discussed recently [21]. It is probable that both hydroxyl radicals and ferryl iron species (also termed crypto-hydroxyl radicals) are formed in relative amounts depending on the actual system, as has recently been suggested [16].

3. Cleavage of peroxides

Besides reductive cleavage of peroxides by iron(II)-species (Eqs. (1) and (2)) peroxides may also be cleaved by iron(III) species:

$$Fe^{3+} + H_2O_2 \rightarrow FeO^{2+} + HO^{\bullet} + H^{+} \quad (3)$$

or in analogy with Eq. (2), as a two-electron transfer reaction with an iron–oxo species (an iron(V) or perferryl species) as reaction product:

$$Fe^{3+} + H_2O_2 \rightarrow FeO^{3+} + H_2O \quad (4)$$

The presence of hydrogen peroxide is accordingly critical for biological systems, and the mechanism and extent of H_2O_2-formation has been extensively studied (see, e.g., the review by Halliwell et al. [25]). $O_2^{\bullet -}$ is readily formed, e.g., in autoxidation of various compounds such as ascorbate [26] or oxymyoglobin [27], and its disproportionation is catalyzed by the enzyme superoxide dismutase to yield hydrogen peroxide [28], or non-enzymatic through disproportionation of the conjugate acid $HO_2^{\bullet}$ ($pK_a = 4.8$) [29,30]. In meat, non-enzymatic formation of H_2O_2 is favored compared to the in vivo conditions due to lower pH and hence higher $HO_2^{\bullet}/O_2^{\bullet -}$-ratios. Contrarily, a gradually *post mortem* decrease of superoxide dismutase activity should lead to a lower enzymatic formation of H_2O_2 in meat than in vivo. Growth of catalase-negative lactic acid bacteria, favored at low pH, may also be important as these bacteria produce H_2O_2 [4].

Not only hydrogen peroxide but also organic peroxides such as the lipid hydroperoxides (LOOH) react with iron species:

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO^{\bullet} + OH^{-} \quad (5)$$

$$Fe^{2+} + LOOH \rightarrow FeO^{2+} + LOH \quad (6)$$

$$Fe^{3+} + LOOH \rightarrow FeO^{2+} + LO^{\bullet} + H^{+} \quad (7)$$

$$Fe^{3+} + LOOH \rightarrow FeO^{3+} + LOH \quad (8)$$

The lipid alkoxyl radical, $LO^{\bullet}$, is reactive and able to abstract a hydrogen atom from another lipid hydroperoxide accelerating lipid oxidation through additional propagation cycles, see Fig. 1. The reactions of Eqs. (5)–(8) depend on the existence of preformed LOOH, and this route of oxidation has accordingly been termed "LOOH-dependent oxidation" [31]. Refined sources of lipids and lards as well as biological membranes always contain at least traces of LOOH formed enzymatically or photochemically [31,32], and a differentiation between LOOH-independent (Eqs. (1)–(4)) and LOOH-dependent (Eqs. (5)–(8)) lipid oxidation is usually difficult.

4. Mechanisms of iron catalysis

The cleavage of peroxides by iron species may be oxidative or reductive, and iron becomes catalytic active although different iron species do not catalyze peroxide cleavage with similar efficiency. The suggested catalytic mechanisms for different iron species will be presented without a detailed evaluation of their relative importance. With few exceptions all mechanisms fit into one of the four groups in Fig. 5.

4.1. Non-heme-iron catalysis

The ligands, which bind to non-heme-iron under the actual conditions, as shown in one example in Fig. 3 are important for the redox-activity and ability to act as a catalyst [13,15]. A general scheme for the catalytic activity of non-heme-iron species is given in Fig. 6.

Compounds which are either reduced by non-heme Fe(II) or oxidized by non-heme Fe(III) will fit into the scheme of Fig. 6 for non-heme-iron catalysis. However, for efficient

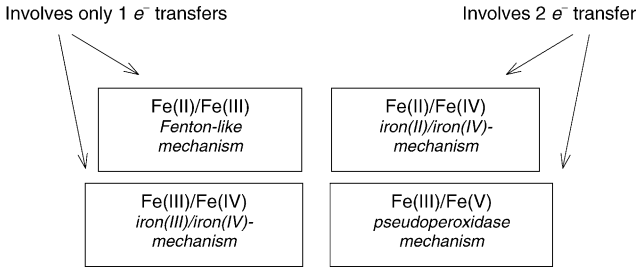

Fig. 5. Iron catalysis mechanisms for oxidation systematized as one- or two-electron transfers and with Fe(II) or Fe(III) as reductant. Redox states involved in the first reaction of the mechanisms are shown.

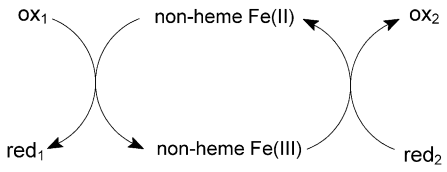

Fig. 6. General mechanism of non-heme-iron catalysis.

catalysis to occur both half reactions must have a reasonable rate. As an example, the formation of hydroxyl radicals through Fenton chemistry is likely to be catalyzed by some non-heme-iron species. Eq. (1) will be the first reaction in Fig. 6 (where H_2O_2 (ox_1) is reduced to $^{\bullet}OH$ and OH^{-}), but as the second reaction of Fig. 6, the oxidation of H_2O_2 by non-heme Fe^{3+} is in most cases too slow to allow efficient catalysis. Instead other half reactions with higher reaction rates become important, and molecules to enter the second half reaction (as red_2) more efficiently than H_2O_2 are ascorbate and superoxide, which are oxidized to ascorbate radicals and oxygen, respectively [26]. Consequently, *the Fenton* reaction (Eq. (1)) is occasionally said to be *superoxide-* or *ascorbate-driven*, and the latter event is regarded as a significant prooxidative activity of ascorbate, which under certain conditions, such as in beer [33], more than counteracts the antioxidative activity of the molecule [26].

Other peroxides than H_2O_2 may react with iron-species, and non-heme-iron catalyzed cleavage of lipid hydroperoxides and protein peroxides is generally assumed to take place according also to the scheme of Fig. 6 [31]. However, a reaction between non-heme-iron species and peroxides located on macromolecules is likely to be influenced be steric factors, polarity differences and actual localization of the reactants within cellular compartments [34]. Non-heme-iron species will thus mainly be located in the aqueous phase, while, e.g., lipid peroxides are located with lipids or in membranes (in the water–lipid interface), cf. Fig. 4. The mechanism of non-heme-iron catalysis of Fig. 6 is only valid for one-electron transfers. If two-electron transfer becomes significant, the mechanism indeed becomes more complex [16,21].

4.2. Heme-iron catalysis

Different mechanisms have been suggested for the catalysis of oxidation by heme-iron, based on studies with mainly myoglobin and hemoglobin, but also with non-protein bound heme-iron. Non-protein bound heme-iron may be released from myoglobin or hemoglobin under certain conditions, and heme (ferroprotoporphyrin) is in solution mainly found as hematin (ferriprotoporphyrin hydroxide). Heme, hematin and hemin are normally used interchangeably to describe the existence of non-protein bound heme-iron (or "free heme-iron"). Hemin is ferriprotoporphyrin chloride, which readily converts to hematin in aqueous solution, and accordingly the term hematin should be used for non-protein bound heme-iron.

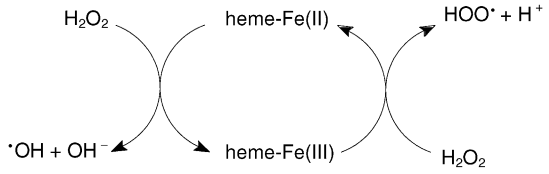

Fig. 7. Proposed mechanism of heme–Fe(II)/heme–Fe(III) catalysis. A Fenton-like mechanism.

4.2.1. Fenton-like mechanism

A Fenton-like mechanism of catalysis is sometimes suggested to account for at least some of the prooxidative activity of heme-iron species [34–36]. A hydroxyl radical, or in the case of LOOH reduction LO•, is formed in the first reaction of the catalytic cycle in Fig. 7. This reaction is thermodynamically favorable as the reduction potential of heme–Fe(III)/heme–Fe(II)-couples is 0.05–0.15 V [37]. Reduction of heme–Fe(III) by H_2O_2 is sometimes proposed as the second reaction of the catalytic cycle in Fig. 7, although it is slower than the first reaction. Even ascorbate as reductant rather than H_2O_2 will not result in very efficient regeneration of heme–Fe(II), as judged from the reduction potentials for the redox couples (0.28 V for asc•/asc$^-$-couple and 1.06 V for HOO•/H_2O_2-couple [38]), and from the rate of heme–Fe(III) reduction by ascorbate, which was found to be slow for the case of myoglobin ($1.2 \times 10^{-2}\,M^{-1}\,s^{-1}$ at pH 7.2, 25 °C [39]).

The heme-iron catalysis through the Fenton-like mechanism is also influenced by the high oxygen affinity of myoglobin and hemoglobin, which generally results in rapid oxygenation of MbFe(II) and HbFe(II) at normal physiological oxygen concentrations [40]. Deoxyheme–Fe(II) proteins available for Fenton-like chemistry is thus dependent on the oxygen dissociation rate constant of the heme–Fe(II)O_2 protein at the actual oxygen pressure.

4.2.2. Heme–Fe(III)/heme–Fe(IV) mechanism

Oxidation of heme–Fe(III) to iron species of higher oxidation states (hypervalent iron) is well established to occur in reactions with peroxides [41–43], and the visible absorption spectra of hypervalent forms of myoglobin and hemoglobin are characteristic and easily distinguished from absorption spectra of the Fe(II)- or Fe(III)-oxidation states of the heme proteins [41,42].

In the mechanism shown in Fig. 8 one-electron transfer takes place between heme–Fe(III) and LOOH. In the initial reaction of the mechanism one electron plus an oxygen atom (to be denoted as O$^-$) is transferred from a peroxide to the heme group and the last electron necessary for the iron-oxygen bond is formally from Fe(III), which is oxidized to Fe(IV) [44].

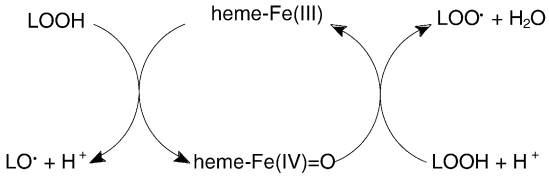

Fig. 8. Proposed mechanism of heme–Fe(III)/heme–Fe(IV)=O catalysis.

Regeneration of heme–Fe(III) in the second reaction of the mechanism is possible as the heme–Fe(IV)=O species is strongly oxidizing. Ferrylmyoglobin (MbFe(IV)=O) thus has a reduction potential for the MbFe(IV)=O/MbFe(III)-couple, which has been estimated to 0.85 V (pH 7.0) [45], and oxidative cleavage of LOOH to LOO• by MbFe(IV)=O as shown in Fig. 8 is favorable [46,47], at least under conditions as found in meat. Also, a wide range of compounds including plant phenols and other antioxidants efficiently react with MbFe(IV)=O and regenerate MbFe(III) [48–50]. Even in the absence of reducing compounds, regeneration of MbFe(III) occurs due to a so-called autoreduction of MbFe(IV)=O (an electron is transferred from the protein moiety to the heme-iron of myoglobin) [51], albeit the rate of autoreduction is probably too slow for efficient catalysis to occur.

In Fig. 8 LOOH is included as oxidant with LO• as the reaction product, which along with heme–Fe(IV)=O has the potential of accelerating oxidation processes [23,38]. MbFe(IV)=O has been detected in model systems upon incorporation of both LOOH and H_2O_2 with MbFe(III) [52–54]. For H_2O_2, •OH and not LO• is the free radical reactive reaction product. The reaction between HbFe(III) and LOOH is less investigated, and detection of a heme–Fe(IV)=O species does not necessarily mean that a cyclic heme–Fe(III)/heme–Fe(IV) conversion as shown in Fig. 8 accounts for the actual mechanism, as will be further discussed below.

4.2.3. Pseudoperoxidase mechanism

In the pseudoperoxidase mechanism, a two-electron transfer from heme–Fe(III) takes place, and subsequently heme–Fe(III) is regenerated by receiving an electron from each of two electron donors. As these donors may be lipids or proteins (or peroxides hereof), the catalytic cycle may both initiate and propagate oxidation [55]. Contrarily, if the donors are antioxidants, relative unreactive radicals are formed, and the overall effect of the catalytic cycle will be antioxidative as a peroxide is removed [55]. Fig. 9A shows the pseudoperoxidase cycle with myoglobin as the heme-iron catalyst and hydrogen peroxide as the peroxide.

In the two-electron transfer reaction (reaction 1 in Fig. 9A) an oxygen atom from the peroxide binds to the heme-iron and the two electrons for the iron–oxygen bond are formally originating from the heme-iron species [44]. One electron formally comes from Fe(III), which is oxidized to Fe(IV), and the other comes from the porphyrin ring, which is oxidized to a porphyrin radical cation [43,56]. For myoglobin and hemoglobin, this radical cation immediately oxidizes an amino acid residue of the surrounding peptide chain, leaving the perferryl species as a protein radical with iron in the oxidation state +4 [56]. The reactions of the pseudoperoxidase cycle are generally the same as the

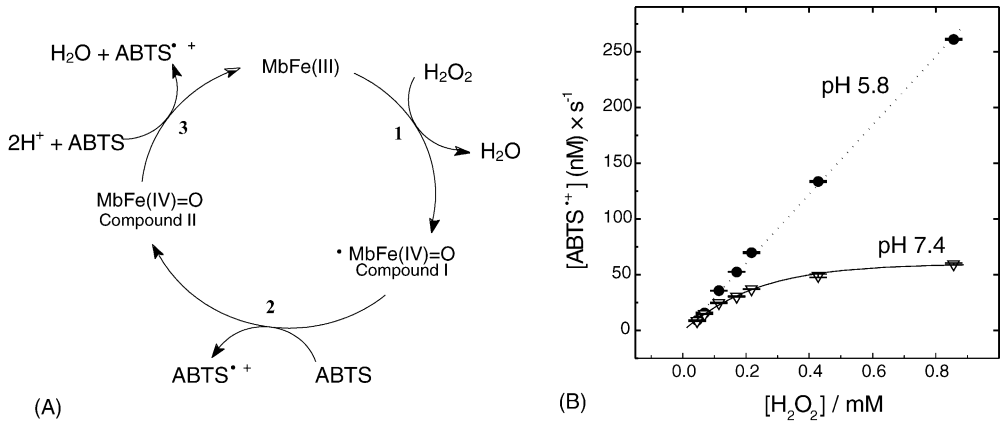

Fig. 9. (A) Pseudoperoxidase cycle of myoglobin. H_2O_2 and ABTS ((2-azino-bis(3-ethylbenzthiazoline-6-sulfonate)) is included as substrates. (B) Peroxidase activity of MbFe(III) as measured by a classical peroxidase assay using ABTS as oxidation substrate. Rate of formation of $ABTS^{\bullet+}$ as a function of $[H_2O_2]$ at 25.0 C in mixtures of 0.50 μM MbFe(III), 1.0 mM ABTS and 0.025–0.850 mM H_2O_2 in 50 mM aqueous MES- or phosphate-buffer (modified from [84]).

reactions undergone by true heme peroxidases [57]. However, myoglobin and hemoglobin react considerably slower with peroxides ($\sim 10^2\ M^{-1}\ s^{-1}$) than classical peroxidases ($\sim 10^7\ M^{-1}\ s^{-1}$) [21,58], and moreover their surrounding peptide chains may be oxidatively modified during repeated peroxidase cycling (due to the protein radical formed in the perferryl state) [41]. Therefore, peroxidase cycling of myogobin and hemoglobin is best described as a pseudoperoxidase activity.

Most studies on pseudoperoxdase activity have been performed with myoglobin, and even though HbFe(III) is also reported to react with H_2O_2 and oxidize to perferryl and ferryl states [42,53], only incomplete information exists for the rate of activation of HbFe(III) and for the reactivity of hypervalent Hb states [59].

Structural differences between the heme pockets of myoglobin and real peroxidases are generally suggested to account for the observed difference in peroxidase activity. The Fe(III)-state of peroxidases (the resting state) is pentacoordinated, i.e. the sixth coordination site is not occupied by any ligand, whereas the Fe(III)-state of myoglobin is hexacoordinated with a water molecule as the sixth ligand. Hence, in contrast to the case of a peroxidase, a water molecule must leave the heme-complex of MbFe(III) before it is oxidized to hypervalent states. Interestingly in this respect, a modified MbFe(III) species being pentacoordinated has been shown to react much faster with H_2O_2 than native MbFe(III) [60]. Nevertheless, pentacoordinated MbFe(II) reacts only a factor 10 faster with H_2O_2 than MbFe(III) does [58,61] (see Section 4.2.4 for details on two-electron reactions of heme–Fe(II) species and peroxides), and hexacoordination of MbFe(III) seems not to be the only reason of myoglobin's moderate peroxidase activity. Numerous studies of myoglobin mutants have been carried out to explore the role of specific amino acid residues on the redox properties of heme proteins, see e.g. [62,63]. Most peroxidases have charged amino acid residues close to the heme-group in the heme-pocket, and these are believed to favor the reactions of the peroxidase cycle through hydrogen bonding and protonisation [57]. Myoglobin does, however, not contain any charged amino acid residues in the heme pocket, and it has been shown that when amino acids with charged side chains are substituted into the heme pocket, the peroxidase activity of myoglobin is at least in some cases enhanced [62,63].

A consequence of the heme-binding environment in myoglobin seems to be a less specific nature of its reaction with peroxides compared to peroxidases, and myoglobin has been shown to react with H_2O_2 in both one- and two-electron transfers reactions, i.e. the first reactions of the heme–Fe(III)/heme–Fe(IV)=O mechanism (Fig. 8) and the pseudoperoxidase mechanism (Fig. 9), respectively [64,65]. Additionally, a range of reactions between $^{\bullet}$MbFe(IV)=O/MbFe(IV)=O and peroxides have been reported (so-called side reactions) [41,58], in effect complicating the overall reaction of myoglobin and peroxides.

To determine whether one- or two-electron transfer dominates the reaction between heme–Fe(III) and a peroxide is not straightforward, because the stable reaction products may be identical for both pathways. In the case of two-electron transfer, perferryl species are formed, but the rapid decay to ferryl species even in the absence of reactants impedes their detection, although it is possible by ESR spectroscopy using a freeze-quenching technique [66]. (Simple UV–vis spectroscopy cannot be used to detect the perferryl state of hemoglobin or myoglobin, as the absorption spectra of the perferryl and ferryl states are identical [41].) A further complication is, however, that $^{\bullet}$OH (and to a lesser extent $RO^{\bullet}$) formed in the case of one-electron transfer readily attacks the protein moiety of the heme species, and thus a ferryl protein radical is formed indirectly and not through the perferryl intermediate [64]. Consequently, both one- and two-electron transfer may lead to formation of perferryl species. Thus, other approaches to differentiate between the two reaction pathways are needed, one being detection of alkoxyl radicals as these are only formed in the heme–Fe(III)/heme–Fe(IV)=O mechanism and not as the

result of the pseudoperoxidase mechanism. Alkoxyl radicals are, however, as already discussed, short-lived species and suitable techniques must be selected to ensure that they are detected immediately upon their formation. A range of studies have used EPR spin-trapping for this purpose [67–69], but recent investigations indicate that spin adducts previous assigned to originate from alkoxyl radicals cannot be definitively differentiated from spin adducts originating from peroxyl radicals, and previous ESR spin-trapping studies should be re-evaluated in this light [70,71].

Adachi et al. [72] and Matsui et al. [65] used a quite simple system by which they upon HPLC analysis of reaction mixtures of heme–Fe(III) and cumene hydroperoxide ($PhC(CH_3)_2OOH$) were able to calculate the ratio between two- and one-electron transfer. Two-electron transfer was assumed to yield cumyl alcohol ($PhC(CH_3)_2OH$) as shown in Eq. (9), whereas one-electron transfer was assumed to yield acetophenone ($PhCOCH_3$) by the reaction sequence shown by Eqs. (10) and (11):

$$\text{MbFe(III)} + \text{PhC(CH}_3\text{)}_2\text{OOH} \rightarrow {}^{\bullet}\text{MbFe(IV)=O} + \text{PhC(CH}_3\text{)}_2\text{OH} \quad (9)$$

$$\text{MbFe(III)} + \text{PhC(CH}_3\text{)}_2\text{OOH} \rightarrow \text{MbFe(IV)=O} + \text{PhC(CH}_3\text{)}_2\text{O}^{\bullet} + \text{H}^{+} \quad (10)$$

$$\text{PhC(CH}_3\text{)}_2\text{O}^{\bullet} \rightarrow \text{PhCOCH}_3 + {}^{\bullet}\text{CH}_3 \quad (11)$$

The reaction of Eq. (11) is of utmost importance for the mechanism assignment because the elimination of a methyl radical ($^{\bullet}CH_3$) from the cumyloxy radical ($PhC(CH_3)_2O^{\bullet}$) occurs instantaneously [73], and therefore the amount of acetophenone in the reaction mixture is proportional to the amount of cumyloxyl radicals formed by one-electron transfer. A ratio of 3.3 for cumyl alcohol/acetophenone (two-electron transfer/one-electron transfer) was determined for the reaction between MbFe(III) and cumene hydroperoxide at pH 7.0 [65], and hence ∼75% of the MbFe(III) molecule was assigned to react through two-electron transfer.

Allentoff et al. [64] found that ∼67% of MbFe(III) reacted in two-electron transfer when the peroxide to be reduced was 4-hydroperoxy-4-methyl-2,6-di-*tert*-butyl-cyclohexa-2,5-dien-1-one (BHTOOH). This was based on HPLC analysis of peroxide reactions products, although an evaluation of the results was somewhat complicated due to a range of degradation products from the cyclic alkoxyl radical formed in one-electron transfer. Recently, Egawa et al. [56] estimated the ratio between two-electron transfer/one-electron transfer for the reaction between MbFe(III) and H_2O_2 to be <0.5, an estimate based on fitting of a complex reaction model to rate data for thioanisole oxidation in the presence of H_2O_2 and MbFe(III). This very low percentage of two-electron transfer is quite surprising, and very different from results obtained in experiments with small organic peroxides [64,65]. Future studies are needed to clarify the consistency of the result for thioanisole oxidation by Egawa et al. [56], and also the steric effect of larger peroxides on the ratio of two- and one-electron transfer reactions with heme–Fe(III) species should be explored. The only safe conclusion at present seems to be that MbFe(III) concomitantly reacts with peroxides in both one- and two-electron transfer reactions as outlined in Fig. 10, and that similar ratios between two- and one-electron transfer cannot be expected for different types of peroxides. Upon mild proteolysis of myoglobin by pepsin, the catalytic activity of myoglobin increases significantly. However, in contrast to cytochromes for which several microperoxidases have been characterized, myoglobin seems to become catalytic by a Fenton-type Fe(II)/Fe(III) mechanism [5].

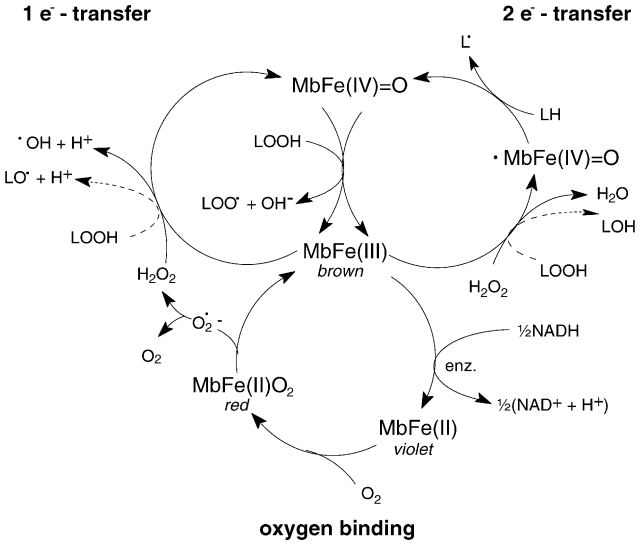

Fig. 10. Metmyoglobin involvement in one- and two-electron transfer processes in lipid oxidation.

4.2.4. *Heme–Fe(II)/heme–Fe(IV)-mechanism*

Heme–Fe(II) species can also be oxidized by two-electron transfer when mixed with peroxides, as indicated by the detection of MbFe(IV)=O in reaction mixtures of MbFe(II) and H_2O_2 [61]. The oxygenated forms of the heme pigments have also been reported to form the ferryl states upon reaction with H_2O_2 [74,75], but results from the study on myoglobin by Yusa and Shikama [61] indicate that $MbFe(II)O_2$ seems to be deoxygenated prior to its reaction with H_2O_2:

$$\text{MbFe(II)O}_2 \rightleftharpoons \text{MbFe(II)} + \text{O}_2 \quad (12)$$

$$\text{MbFe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{MbFe(IV)=O} + \text{H}_2\text{O} \quad (13)$$

The overall rate constants for oxidation of MbFe(II) and $MbFe(II)O_2$ by H_2O_2 was estimated to be $3.5 \times 10^3\ M^{-1}\ s^{-1}$ and $20.8\ M^{-1}\ s^{-1}$, respectively, at pH 7.0 (25 °C), and the difference in oxidation rates is in agreement with the low fraction of MbFe(II) in the myoglobin/H_2O_2 solutions at atmospheric oxygen pressure due to the strong binding of O_2 to MbFe(II), as evidenced from the value for the dissociation constant,

$K_D = 1.64 \times 10^{-6}\ M^{-1}$ at 25 °C. However, it should be noted that the rate constants found in the study of Yusa and Shikama [61] were estimated using very low H_2O_2 concentrations, and that high H_2O_2/Mb-ratios complicates the reaction scheme because of further reactions between reaction products and excess H_2O_2 [61,74].

MbFe(II)/HbFe(II) must be regenerated from MbFe(IV)=O or HbFe(IV)=O, respectively, if a catalytic reaction cycle should be functioning (i.e. a catalytic heme–Fe(II)/heme–Fe(IV)-mechanism). Such regeneration does, however, not seem to occur, as the ferryl states preferentially reduce to ferric states in one-electron transfer reactions. Actually, mixtures of MbFe(II)/MbFe(II)O_2 and H_2O_2 are rapidly converted into MbFe(III), because MbFe(IV)=O reacts with MbFe(II)O_2/MbFe(II) in a so-called synproportionation reaction [61,76]:

$$\text{MbFe(IV)=O} + \text{MbFe(II)} + 2H^+ \rightarrow 2\text{MbFe(III)} + H_2O \quad (14)$$

A synproportionation reaction has also been shown to take place for hemoglobin [75], and generally synproportionation accelerates the rate of reduction of the heme–Fe(IV)=O species relative to autoreduction [75,76]. Nevertheless, in biological systems a heme–Fe(IV)=O reduction can also be accelerated by other reducing agents than heme–Fe(II) species, such as e.g. ascorbate [75,77].

In conclusion, a catalytic heme–Fe(II)/heme–Fe(IV)-mechanism does not seem to operate, because heme–Fe(II) is not regenerated when heme–Fe(III) is the "end-product". Nevertheless, the formation of heme–Fe(III) in reactions between heme–Fe(II) and peroxides does not as such prevent heme-iron catalysis from occurring, since MbFe(III) may exert catalytic activity through the mechanisms previously described (see Figs. 8 and 9). In agreement with this view, Reeder et al. [78] found less $^{\bullet}$MbFe(IV)=O-induced protein crosslinking in a solution of MbFe(II)O_2 than in a solution of MbFe(III) upon additions of low concentrations of H_2O_2. This was readily explained by the fact that $^{\bullet}$MbFe(IV)=O is not formed in the reaction between MbFe(II)O_2 and H_2O_2, but only by reaction of MbFe(III) and H_2O_2. However, for addition of increasing amounts of H_2O_2, the $^{\bullet}$MbFe(IV)=O-induced cross-linking increased by the same extent in both the MbFe(II)O_2-solution and the MbFe(III)-solution [78]. Thus, at high H_2O_2-concentrations no limitation on further reactions between MbFe(III) (formed upon synproportionation in the MbFe(II)O_2-solution) and H_2O_2 exists, and increasing amounts of H_2O_2 leads to higher formation of $^{\bullet}$MbFe(IV)=O, also in a MbFe(II)O_2-solution.

The reaction sequence of Eqs. (12)–(14) has been suggested to have antioxidative and protective effects because H_2O_2 is removed and the intermediate ferryl species is rapidly removed [75,76]. As can be rationalized from the experiment of Reeder et al. [78], this may be correct at low hydrogen peroxide concentrations. Contrarily, when peroxide concentration increases, the antioxidative effect of the reaction sequence is questionable, and depends on the pro/anti-oxidative effect of subsequent reactions between formed MbFe(III) and H_2O_2.

A peroxide not previously mentioned is peroxynitrite ($ONOO^-$), which is rapidly generated from nitrogen oxide (NO) and $O_2^{\bullet -}$ ($k \sim 10^9\ M^{-1}\ s^{-1}$) [21]. Neutral and acidic solutions of $ONOO^-$ are unstable because the conjugated acid, peroxynitrous acid (HOONO) decomposes [21,79]. However, $ONOO^-$/HOONO as well as some of their decomposition products are capable of oxidizing biomolecules [79]. It is well established that MbFe(II)O_2/MbFe(II) is converted to MbFe(III) upon mixing with $ONOO^-$/HOONO [80–82], and it has been shown that MbFe(IV)=O is an intermediate in this conversion [82]. Thus MbFe(II)O_2/MbFe(II) react with $ONOO^-$/HOONO in two-electron transfer reactions corresponding to the reaction with H_2O_2 in Eq. (13) [82,83]. Remarkably, the rate constants for oxidation of MbFe(II) and MbFe(II)O_2 to MbFe(IV)=O by HOONO were estimated to $\sim 10^6\ M^{-1}\ s^{-1}$ and $5 \times 10^4\ M^{-1}\ s^{-1}$, respectively, at pH 7.4 (25 °C) [82]. These values are three orders of magnitude higher than the corresponding reactions with H_2O_2 (see above), and accordingly the significance of reactions between heme–Fe species and peroxynitrite should be further explored. Notably in this respect, MbFe(III) has been reported not to react with $ONOO^-$/HOONO [83].

5. The reactivity of heme Fe(IV)=O

The pH dependence of the rate of ferrylmyoglobin reduction has been investigated by use of various reducing compounds [49,51,77,84], and characteristic pH-profiles have been obtained, which could be accounted for by kinetic models including an acid–base equilibrium of ferrylmyoglobin:

$$\text{MbFe(IV)=O, H}^+ \rightleftharpoons \text{MbFe(IV)=O} + H^+ \quad (15)$$

MbFe(IV)=O,H^+ is a protonated ferrylmyoglobin species, for which the location of the proton is unknown. The effective charge of the reaction center in MbFe(IV)=O in a physiological chloride medium was found to be +1.0 from an analysis of the kinetic salt effect on the specifically acid catalyzed autoreduction of MbFe(IV)=O using the Bjerrum–Brøndsted theory [51]. This is the same effective charge as found for the iron center in MbFe(II)O_2 from the kinetic salt effect for acid catalyzed autoxidation of MbFe(II)O_2 [85]. The protonated form, MbFe(IV)=O,H^+, was found to have a pK_a-value of 4.9, 5.0, 5.2 and 5.3 in kinetic investigations of ferrylmyoglobin reduction by NADH [51], chlorogenate [49], ABTS (2-azino-bis(3-ethylbenzthiazoline-6-sulfonate)) [84] and ascorbate [77], respectively. The similar estimates of the pK_a for Eq. (15) for different reductants are in agreement with two reactive populations of ferrylmyoglobin and in agreement with the assumption, that only one proton is bound to the heme cavity close to

the reaction center. The proton may increase the reactivity of the hypervalent iron by a simple increase in positive charge, which will facilitate electron transfer from the reductant, or through some not yet fully elucidated binding mechanism.

The consequence of protonisation of ferrylmyoglobin is an increased reactivity towards reducing substrates. Accordingly, enhanced prooxidative activity may be observed where ferrylmyoglobin formation occurs in systems with lower pH-values. This is due to: (i) increased rate of MbFe(III)-regeneration and thereby increased catalytic activity of MbFe(III) through mechanisms involving the ferryl intermediate; and (ii) increased oxidation of substrate molecules by ferrylmyoglobin (e.g. lipids, proteins and peroxides hereof) into reactive radicals. However, further investigation including systematic comparison of the reactivity of MbFe(IV)=O,H^+ towards lipids and proteins relative to its reactivity towards antioxidants is needed, also in order to understand the role of long-lived protein radicals. Conflicting results have been obtained for the pH-dependence of ferrylmyoglobin reactivity towards lipid hydroperoxides. Baron et al. [47] reported a different pH-dependency, with a slightly increased reactivity of ferrylmyoglobin towards LOOH at increasing pH, whereas Reeder & Wilson [53] obtained a pH-dependency of LOOH-reduction by ferrylmyoglobin consistent with the hypothesis of MbFe(IV)=O,H^+ being much more reactive than MbFe(IV)=O.

Reeder and Wilson [53] have recently investigated the pH dependence of the reaction of MbFe(III) or HbFe(III) with H_2O_2, and estimated the rate of ferryl formation and the rate of ferryl reduction in agreement with a heme–Fe(III)/heme–Fe(IV)-mechanism. Interestingly, for both myoglobin and hemoglobin the estimated pH-profiles for ferryl reduction were in agreement with the experimental pH-profiles obtained for ferrylmyoglobin in other studies [49,51,77,84], and Reeder and Wilson [53] also suggested that ferrylmyoglobin is protonised in order to account for the pH-dependence of its reactivity.

In a study of the reaction between MbFe(III) and H_2O_2 in the presence of ABTS, all three reactions of the pseudoperoxidase cycle (Fig. 9A) were examined at pH 7.4 and pH 5.8 [84]. Rate constants for the individual reactions were determined by means of sequential stopped flow spectroscopy, while the overall rate of peroxidase cycling was determined by a classical peroxidase assay (result shown in Fig. 9B). A clear conclusion was that the enhancement of the pseudoperoxidase activity of MbFe(III) at low pH is mainly due to protonisation of ferrylmyoglobin, which change the rate limiting reaction of the peroxidase cycle from ferrylmyoglobin reduction (reaction 3 of Fig. 9B) to MbFe(III) activation by H_2O_2 (reaction 1 of Fig. 9B). Contrarily, the rates of reaction 1 and 2 of the peroxidase cycle were found to be insensitive to a decrease in pH from 7.4 to 5.8.

The consequence of increased ferrylmyoglobin reactivity at decreasing pH in tissue depends on the nature of the molecules which is oxidized along with the reduction of ferrylmyoglobin to MbFe(III). For both a heme–Fe(III)/heme–Fe(IV)-mechanism and the pseudoperoxidase mechanism the formed hypervalent heme species will be deactivated without formation of harmful radicals in the presence of effective antioxidants. Likewise, for both mechanisms a faster depletion of available antioxidants is expected at decreasing pH due to MbFe(IV)=O,H^+-mediated increase in the rate of catalytic cycling. However, for the heme–Fe(III)/heme–Fe(IV)-mechanism peroxides are cleaved into reactive alkoxyl radicals, and hence one may speculate that more severe oxidative damage is the result for conditions which favor the heme–Fe(III)/heme–Fe(IV)-mechanism relative to the pseudoperoxidase mechanism.

The method developed by Matsui et al. [65] (see Eqs. (9)–(11)), to distinguish between two-electron transfer and one-electron transfer based on mixing of MbFe(III) and cumene hydroperoxide should be used to characterize the balance between the two mechanisms at decreasing pH. A lowering of the ratio between two-electron transfer and one-electron transfer upon a pH-decrease would indicate that a heme–Fe(III)/heme–Fe(IV)-mechanism becomes more dominating at lower pH relative to the pseudoperoxidase mechanism, provided that other peroxides react comparable to cumene hydroperoxide.

6. Nitric oxide and iron catalysis

Under normal homeostasis NO is generated in nanomolar quantities and acts primarily as a vasodilator and signal transmitter in the nervous system. During conditions of oxidative stress or due to activated macrophages, NO is in some cases present at higher concentrations in vivo and it seems uncertain whether the net result is prooxidative or antioxidative.

6.1. NO-induced oxidation of oxymyoglobin

The second order rate constant for the reaction of NO with MbFe(II)O_2 (Eq. (16)) has been found at pH 7.0 to be around $4 \times 10^7\ M^{-1}\ s^{-1}$, and HbFe(II)$O_2$ has been shown to react even faster [86,87].

$$MbFe(II)O_2 + NO \rightarrow MbFe(III) + NO_3{}^- \quad (16)$$

Doyle and Hoekstra were the first to suggest peroxynitrite as an intermediate in the NO-induced oxidation of oxygenated Hb/Mb, and that an inner-sphere electron transfer process was most likely to occur as NO easily access the heme cavity [88]. A reaction intermediate has also been detected for NO-induced oxidation of MbFe(II)O_2 or HbFe(II)O_2, and the intermediate is suggested to be a peroxynitrito complex of Hb/Mb, formed as NO attacks the O_2 ligand [87,89]. The kinetics of the reaction is found to depend on pH of the solvent, as alkaline pH tends to stabilize the intermediate peroxynitrito complex [89]. Studies of the mechanism including site-directed mutants of myoglobin point towards the entry of NO into the heme cavity as a rate-determining step [86]. The re-

action of oxygenated heme proteins, mainly hemoglobin, and free NO is considered so efficient and rapid that it is widely used as a standard method for detection and quantification of NO [90,91].

6.2. Reactions of NO and other small activated compounds

The reaction between NO and the superoxide anion ($O_2^{\bullet -}$) is nearly diffusion controlled:

$$NO + O_2^{\bullet -} \rightarrow ONOO^- \quad (17)$$

The product peroxynitrite, $ONOO^-$, is a strong oxidant, which has the capacity to initiate lipid oxidation [92] or cause oxidative protein modification [93], e.g. nitration of tyrosine residues in proteins, or it may isomerise to nitrate. However, the reactivity of $ONOO^-$/ONOOH is highly pH-dependent [79], and where the anion is quite stable at pH equal or above $pK_a = 6.8$, the isomerisation to the less reactive nitrate will dominate at low pH [94]. This pH-dependent behavior of $ONOO^-$ reactivity together with generally high second order rate constants for its reaction with biomolecules [95], and the unusually rapid permeation across membranes observed for the protonised form ONOOH [96], allows it to cause considerable damage to lipids and proteins. The formation of 3-nitrotyrosine has been used as a marker of peroxynitrite formation, and an indicator of oxidative stress involving reactive nitrogen oxide species in biological systems [97,98]. However, recent studies have shown that proteins such as hemoglobin and myoglobin in presence of nitrite and H_2O_2 may cause nitrosation of the heme protein or of other proteins [99,100], and caution needs to be taken when assessing studies reporting deleterious pathways of NO chemistry in the presence of H_2O_2 and heme proteins. Carbon dioxide seems to mediate a catalytic effect on $ONOO^-$ reactivity [101,102], as an adduct is formed (Eq. (18)):

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \quad (18)$$

The second order rate constant for adduct formation is $3.0 \times 10^4\ M^{-1}\ s^{-1}$, which, given the relative high CO_2 concentration in e.g. venous blood, facilitates this reaction. However, whether this adduct will enhance the harmful effect of $ONOO^-$ or protect from cellular damage is disputed. Indeed most studies report an increased rate of decomposition of $ONOOCO_2^-$ into NO_3^- with catalytic regeneration of CO_2 [103], but others find substantial degree of homolytical cleavage of the adduct to yield both carbonate ($CO_3^{\bullet -}$) and nitrogen dioxide ($^{\bullet}NO_2$) radicals [104].

NO reacts rapidly with a number of free radicals, including hydroxyl ($OH^{\bullet}$) (Eq. (19)) and alkyl ($L^{\bullet}$), alkoxyl ($LO^{\bullet}$) or peroxyl radicals ($LOO^{\bullet}$) (Eq. (20)), and for the reactions with $^{\bullet}OH$ and $LOO^{\bullet}$ very high second order rate constants of $10^{10}\ M^{-1}\ s^{-1}$ and $2 \times 10^9\ M^{-1}\ s^{-1}$, respectively, have been reported [105]:

$$NO + {}^{\bullet}OH \rightarrow HNO_2 \quad (19)$$

$$NO + LOO^{\bullet} \rightarrow LOONO \quad (20)$$

$ONOO^-$ is most likely formed in muscle food by enzyme systems generating NO and $O_2^{\bullet -}$, and $ONOO^-$ may act as a potential initiator of lipid and protein oxidation or simply deplete naturally occurring antioxidants [106]. $ONOO^-$ has been shown in the presence or absence of CO_2 to promote oxidative processes, resulting in an increasing concentration of both primary and secondary lipid oxidation products [107].

6.3. Reaction of $ONOO^-$ with heme proteins

$ONOO^-$ causes rapid conversion of MbFe(II)O_2 to MbFe(III) while presence of CO_2 and lowering of pH seems to reduce MbFe(II)O_2 conversion slightly [81]. In the reaction between MbFe(II)O_2 and $ONOO^-$, $ONOO^-$ was suggested to oxidize the small fraction of MbFe(II), present in equilibrium with MbFe(II)O_2, to hypervalent myoglobin (Eq. (21)), while a subsequent reduction by $ONOO^-$ was found to yield MbFe(III) and the peroxynitrite radical (Eq. (22)). These two consecutive reactions were found to have comparable rates, as the second order rate constants at 20 °C and pH 7.3 are 5.4×10^4 and $2.2 \times 10^4\ M^{-1}\ s^{-1}$, respectively:

$$MbFe(II) + ONOOH \rightarrow MbFe(IV){=}O + HNO_2 \quad (21)$$

$$MbFe(IV){=}O + ONOOH \rightarrow MbFe(III) + OONO^{\bullet} + OH^- \quad (22)$$

It was further found that the rate of the initial reaction (Eq. (21)) significantly increased ($4.1 \times 10^5\ M^{-1}\ s^{-1}$), while the rate of Eq. (22) remained practically unaltered in the presence of 1.2 mM CO_2 [83].

A much more complex reaction pattern has recently been suggested for the reaction of HbFe(II)O_2 and $ONOO^-$ [108]. For this reaction sequence (Eqs. (23)–(25)), the initial reaction step is simply an exchange between $O_2^{\bullet -}$ and $ONOO^-$ in HbFe(III). The second step (Eqs. (24) and (25)) is suggested to be a simple rearrangement of $ONOO^-$ while still coordinated in the heme cavity, thus leading to the release of NO_3^- and HbFe(III). These results support a role of hemoglobin as a relevant intravascular sink for $ONOO^-$. It should be noted, however, that a minor pathway involves a ferric hemoglobin complex of $ONOO^-$, in which the ferric heme-iron catalyses $ONOO^-$ decomposition by homolytic N–O bond cleavage to yield $NO_2^{\bullet}$ and HbFe(IV)=O.

$$HbFe(III)O_2^{\bullet -} + ONOOH \rightarrow HbFe(III){-}ONOO^- + O_2^{\bullet -} + H^+ \quad (23)$$

$$HbFe(III){-}ONOO^- \rightarrow Hb[Fe(III){-}O\cdots NOO^-] \quad (24)$$

$$Hb[Fe(III){-}O\cdots NOO^-] \rightarrow HbFe(III) + NO_3^- \quad (25)$$

Nitrated amino acid residues such as 3-nitrotyrosine in myoglobin or hemoglobin could only be detected in low concentration following exposure to $ONOO^-$, while apoMb

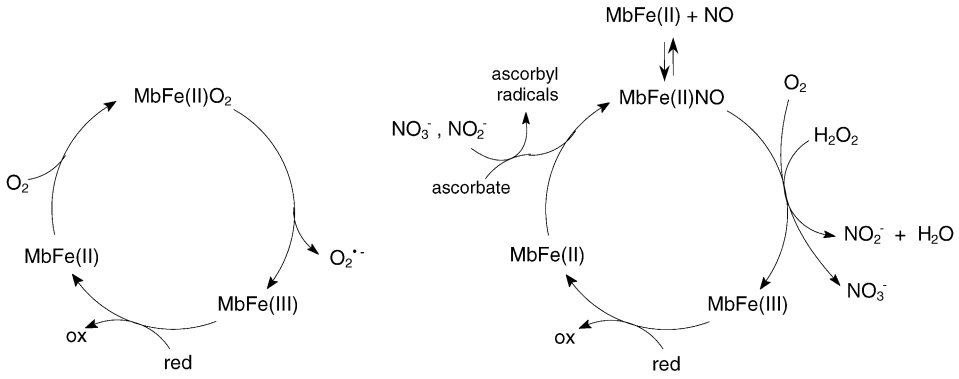

Fig. 11. A comparison between the prooxidative cycling of oxymyoglobin generating superoxide radicals and the antioxidative cycling of nitrosylmyoglobin depleting oxygen or peroxides and with regeneration by various reductants.

or the cyano complex of MbFe(III) had larger yield for 3-nitrotyrosine and even low quantities of nitrated tryptophan, indicating that the heme centre of myoglobin may act as an efficient scavenger of $ONOO^-$ thereby protecting not only its globin part but also other proteins from nitration. This adds further support to the general theory of myoglobin acting as protector of cellular respiration apart from assisting in facilitated diffusion of O_2 in muscle tissue.

Whether NO acts as a pro- or anti-oxidant mainly depends on the concentration of NO and the concomitant presence of $O_2^{\bullet -}$ [109]. Equimolar fluxes of $O_2^{\bullet -}$, H_2O_2 and NO have been found to enhance the oxidation of a substrate (dihydrorhodamine) in a model system containing MbFe(III), while further increases in NO concentration significantly reduced substrate oxidation via inhibition of $^{\bullet}$MbFe(IV)=O/MbFe(IV)=O formation [110]. Addition of NO to leukemic cells exposed to oxidative stress (20 μM Fe^{2+}) inhibited the cells' oxygen consumption, which was used as a measure of lipid peroxidation, in a concentration dependent manner [111]. Another study has proved NO to protect cardiomyocytes against *tert*-butyl hydroperoxide-induced: (i) formation of non-protein and protein-centred free radical species; and (ii) concomitant peroxidation of membrane phospholipids [112], which indicates that NO may act as an antioxidant in heart tissue.

NO and its interaction with heme proteins was early suggested as the active factor in preventing the development of oxidative rancidity in nitrite-cured meat [113,114]. So far, only a single study has investigated the isolated effect of MbFe(II)NO in oxidative processes in a carotene-linoleate model system, and it was found that 2–10 μM of MbFe(III) or MbFe(II)O_2 were prooxidative, while MbFe(II)NO at all investigated concentrations was not prooxidative, and MbFe(II)NO could even inhibit the prooxidative effect of 2 μM MbFe(III) [115]. It has further been found that MbFe(II)NO in the presence of excess MbFe(III) almost completely inhibited oxygen consumption in a peroxidating lipid system with methyl linoleate as substrate [116]. NO has been found to inactivate oxidizing ferrylmyoglobin species [117]. The reaction mechanism includes two steps with a rapid initial formation of a reaction intermediate, nitritomet-myoglobin (MbFe(III)ONO), which subsequently decays on a longer time scale (Eqs. (26) and (27)). The second order rate constant for the reaction between MbFe(IV)=O and NO (Eq. (26)) is found to be $1.8 \times 10^7\ M^{-1}\ s^{-1}$ at pH 7.5 and 20 °C, while the first order rate constant for decay of the reaction intermediate in Eq. (27) is $3.4\ s^{-1}$.

$$[\text{MbFe(IV)=O} \leftrightarrow \text{MbFe(III)–O}^{\bullet}] + \text{NO} \rightarrow \text{MbFe(III)ONO} \quad (26)$$

$$\text{MbFe(III)ONO} \rightarrow \text{MbFe(III)} + \text{NO}_2^- \quad (27)$$

Thus, it seems that free NO may either inhibit or in the presence of $O_2^{\bullet -}$ promote lipid peroxidation, whereas when NO is bound to myoglobin it may act as bioactive reservoir that can be either released to scavenge $OH^{\bullet}$ and lipid derived radicals or in the form of nitrosylmyoglobin deplete O_2 and H_2O_2. The difference between MbFe(II)O_2 as a prooxidant and MbFe(II)NO as an antioxidant should be clear from the comparison made in Fig. 11.

7. Conclusions

Lipid oxidation in biological systems is characterized by a lag-phase, where little if any lipid oxidation products are formed. However, radicals are continuously generated but quenched by various types of substrates. Iron species are well established as responsible for initiation of oxidative processes in tissue. As for muscles, myoglobin is the abundant iron species but also hemoglobin, non-heme-iron and hematin released from myoglobin are important, as has been reviewed by Kanner [118]. In the present review, lipoxygenase activity has only been considered briefly, while the prooxidative activity of simpler iron species in relation to membranes and interphases in emulsions have been related to current efforts to develop emulsifiers for cosmetics, food and pharmaceuticals, which repels prooxidants like iron and attracts antioxidants [9]. The prooxidant activities of heme pigments are strongly

affected by low pH, high prevalence of deoxygenated heme pigment as under ischemic conditions, oxidative modification of the heme pigments and proteolysis, and all these conditions may result in enhancement of their prooxidative capacity. Nitric oxide modifies and in most cases moderates the prooxidative activity of the heme pigments [119]. The mechanisms for prooxidative activity have been classified into four types and the main conclusion of this review is that each of the mechanisms alone or combinations of the mechanisms may operate depending on the specific conditions in biological systems. It is hoped that this attempt of systematization will help to define new experiments for describing the balance between prooxidative and antioxidative activity of heme pigments.

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