Lipid Peroxidation Induced by H_2O_2-Activated Metmyoglobin and Detection of a Myoglobin-Derived Radical

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The aim of this work was to better understand the oxidative processes which regulate lipid and myoglobin oxidation during an 8-day storage period of two bovine muscles: Longissimus lumborum (LL), stable from viewpoint of color stability, and Psoas major (PM), unstable. Lipid peroxidation (TBA-RS accumulation), induced by a H_2O_2-activated MetMb system, was greater in microsomal membranes from Psoas major muscle than from Longissimus lumborum muscle and particularly after 8 days of meat storage. These results could be partly explained by the differences in the phospholipids and fatty acids composition of microsomal fractions of two studied muscles. To better understand these oxidative processes in relation to muscle type and post-mortem storage time, it was measured in vitro, by optical and ESR spectroscopy, the formed radicals in a H_2O_2-activated MetMb system with or without microsomes. By optical spectroscopy, it was shown that the decay rate constant of the ferrylmyoglobin radical into ferric myoglobin was enhanced (6-fold) after addition of the microsomal membranes. No muscle effect was noted. With ESR spectroscopy, it was shown that a tyrosine peroxyl radical was formed. After addition of the microsomal membranes, the increase of the decay rate constant was very low (1.4-fold) but was greater when the microsomal fraction was extracted after 8 days of meat storage.

Keywords: *Meat; phospholipids; myoglobin oxidation; lipid peroxidation; radical; ESR*

INTRODUCTION

At the retail level, the change in color of fresh beef meat from bright red to brown is an important problem for the meat industry because consumers relate the bright red color of meat to freshness and the discoloration of packaged fresh meat is synonymous with unacceptability. Among the numerous factors responsible for meat discoloration during storage and which also contribute to the differences in color stability between beef muscles, the most important is autoxidation of oxymyoglobin to metmyoglobin (Renerre and Labadie, 1993). It is also well established that during meat storage, lipid oxidation is one of the primary causes of quality loss and that oxidation of lipid and myoglobin are interdependent phenomena (Benedict et al., 1975). For Love (1983), lipid oxidation is nonenzymatic with myoglobin being the major catalyst. With liposomes, prepared from muscle lipid extracts, it has also been shown that the phospholipid content and composition of muscles could be implicated in the differences in meat color stability (Genot et al., 1991).

During the autoxidation process, oxymyoglobin separates into metmyoglobin (MetMb) and a free superoxide anion $O^{\bullet-}{}_2$, which is rapidly transformed by dismutation into hydrogen peroxide. Moreover, when provided fully with their substrates, organelles such as mitochondria, microsomes, and peroxisomes are effective H_2O_2 generators and it is well established that enzymic membrane systems catalyze the oxidation of oxymyoglobin (Lin and Hultin, 1977; Anton et al., 1993). In parallel, other studies have demonstrated that the interaction of H_2O_2 with MetMb generated activated MetMb capable of initiating lipid oxidation (Kanner and Harel, 1985a,b). These results were confirmed by different authors such as Rhee et al. (1987) and Asghar et al. (1988). Nevertheless, as noted by Kanner (1992), muscle cytosol contains compounds, such as enzymes (catalase, peroxidase), which control the amount of hydrogen peroxide and may inhibit nonenzymic lipid peroxidation. The reaction of MetMb with H_2O_2 is known to involve the formation of a ferryl (iron(IV)=oxo) species and a protein radical (Rice-Evans et al., 1989; Davies, 1990) which can be identified by ESR spectroscopy.

To better understand these oxidative processes implicated in meat quality, we have measured in vitro, by optical and ESR spectroscopy, the formed radicals in a H_2O_2-activated MetMb system with or without microsomes. The effect of the phospholipid and fatty acid composition of the microsomal membranes of two muscles and the effect of post-mortem storage time on these oxidations is also examined.

MATERIALS AND METHODS

Methods. Young Friesian bulls (3 years old) were purchased from local cattle breeder and slaughtered in the Meat Research Department abattoir. At 1 h post-mortem, two muscles were taken: Longissimus lumborum (LL), stable from viewpoint of color stability (Renerre, 1984), and Psoas major (PM), unstable. These samples were stored at 4 °C for 8 days.

Metmyoglobin Preparation. At 2 h post-mortem (day 0: D0), oxymyoglobin from each type of bovine muscle was purified by ammonium sulfate fractionation and chromatography on HPLC Mono Q column (Pharmacia), as previously described (Gatellier et al., 1993). Metmyoglobin was obtained from oxymyoglobin by chemical oxidation with potassium ferricyanide and dialyzed against a 100 mM sodium phosphate buffer at pH 7. Then, the metal chelating agent, DETAPAC (0.1 mM), was added to the buffer to ensure that trace metals were removed. Myoglobin concentration was measured by absorbance at 540 nm of the cyanmetmyoglobin complex formed by the Drabkin reaction (Drabkin, 1950).

Preparation of H_2O_2-Activated MetMb System. A 30% commercial solution of H_2O_2 (Sigma) was diluted in distilled water to a 8.82 mM stock solution. The concentration of the stock solution was calculated from the molar extinction coefficient of 43.6 M^{-1} cm^{-1} at 240 nm. This stock solution was prepared just before the beginning of the experiment. Aliquots of this stock solution were added to the metmyoglobin solution to initiate the oxidation in vitro.

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Preparation of Microsomal Membranes. Microsomal membranes from the two muscles were prepared at day 0 (D0) and day 8 (D8) of storage, by differential centrifugation method (Anton et al., 1991). The 100 000 g pellet was resuspended in 5 mM histidine and 0.12 M KCl buffer at pH 6.8. Microsomal membrane concentration was assessed by a biuret method (Gornall et al., 1949) for protein determination.

Lipid Peroxidation. Lipid peroxidation in microsomal membranes was measured by incubating 1 mg/mL microsomes at 37 °C with the reaction mixture containing 30 μM metmyoglobin and 30 μM H_2O_2 in 100 mM sodium phosphate buffer at pH 7. The extent of lipid peroxidation after aqueous acid extraction was determined by measuring malondialdehyde formation with the thiobarbituric acid test (TBA), as described by Sunderman et al. (1985), with an incubation time of 24 h. Malondialdehyde formed by peroxidations occurs predominantly in polyunsaturated lipids with three carbons between the double bonds.

Visible Spectra. Visible spectra (460–760 nm) were recorded at room temperature on a Uvikon 860 spectrophotometer using a 10 mm path length cell. The first spectrum was recorded 30 s after adding H_2O_2 to the metmyoglobin solution. Spectra were recorded subsequently every 10 min and decay rates of the signal were measured by following the absorbance at 580 nm. Metmyoglobin and H_2O_2 concentrations were 70 μM. When added, microsomal membranes concentration was 0.21 mg/mL.

ESR Spectra. ESR spectra were recorded on a Bruker ER 200 D spectrometer using 0.1 M DMPO (5,5-dimethyl-1-pyrroline 1-oxide) spin-trapping reagent. DMPO was purchased from Aldrich (97% purity) and was used without further purification. Metmyoglobin was concentrated by ultrafiltration on a Amicon YM 10 membrane and resuspended in the 100 mM sodium phosphate buffer at pH 7, passed previously through a Chelex 100 column. Experimental conditions were gain, 4×10^5; modulation, 1 G; and scan speed, 1 G/s. The magnetic field was set at 3470 G. The first spectrum was recorded 4 min after H_2O_2 addition to the metmyoglobin and DMPO mixture. The spectra were recorded every 3 min. Decay rates were measured by following the 3484 G resonance. Metmyoglobin and H_2O_2 concentrations were 600 μM while microsomes were at a concentration of 1.82 mg/mL. All spectra were recorded at room temperature.

Phospholipids and Fatty Acids Composition. Lipids were extracted from the microsomal membranes by the Folch method (Folch et al., 1957). Phospholipid fractionation was realized by HPLC on a silicic acid column. The quantification was done according to Leseigneur-Meynier et al. (1989). Fatty acid composition of phospholipids was determined by gas chromatography of methyl esters prepared as described by Berry et al. (1965).

Unpaired Student's t-test was performed to ascertain levels of statistical significance. All values are reported as mean ± standard deviation of the mean.

RESULTS AND DISCUSSION

Lipid Peroxidation and Phospholipids Composition. The TBA assay was employed to quantify the aldehydic degradation products of the lipid peroxidation; nevertheless, as this method uses aqueous acid extraction, it is better to speak of thiobarbituric acid reactive substances (TBA-RS) because other substances may also react with TBA (Raharjo and Sofos, 1993). Lipid peroxidation of microsomal membranes alone, at 37 °C and at pH 7, was relatively slow and TBA-RS increased less than 10% after 24 h incubation in both systems (results not shown). Addition of metmyoglobin–H_2O_2 mixture to the microsomal fraction enhanced the rate of the lipid peroxidation (Figure 1a). This data is in good agreement with those of Rice-Evans et al. (1989) and Newman et al. (1991). For these last authors, the accumulation of TBA-RS increased 2-fold when membranes were incubated for 3 h at 37 °C with a metmyoglobin–H_2O_2 mixture. For a 1:20 ratio of MetMb/H_2O_2, addition of membranes to this mixture, after 3 h and in the presence of desferrioxamine, caused a 10-fold increase in TBA-RS accumulation and an iron release of about 14% (Rice-Evans et al., 1989).

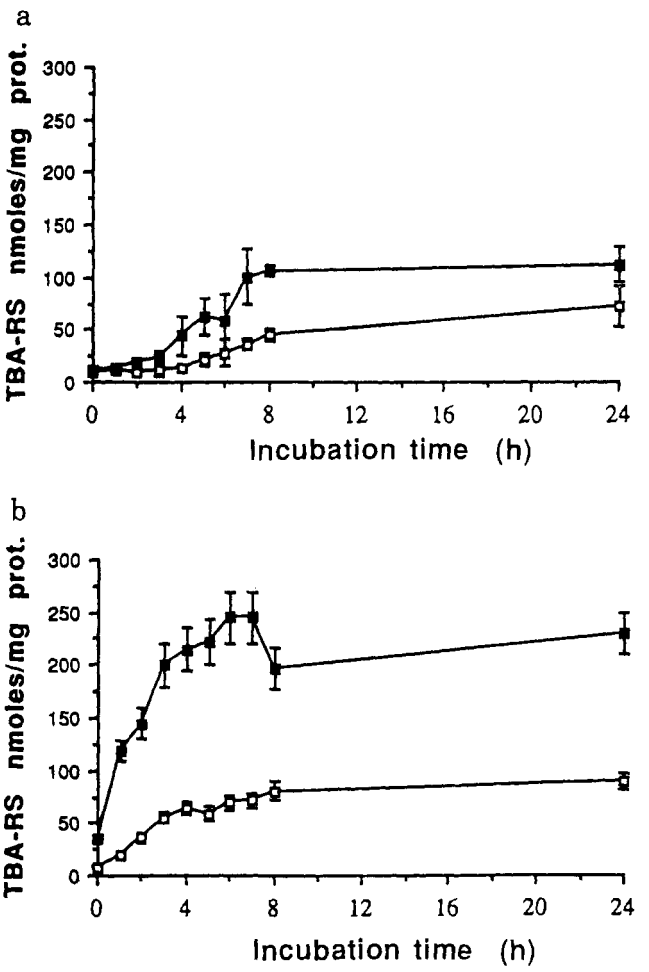

Figure 1. Influence of metmyoglobin (30 μM)–H_2O_2 (30 μM) on microsomal lipid peroxidation as estimated by the TBA test. Microsomes are extracted at day 0 (a) and day 8 (b) from Longissimus lumborum (LL, □) and Psoas major (PM, ■).

When microsomes were extracted at day 0 (D0) (Figure 1a), TBA-RS accumulation was greater in PM than in LL muscle which is a more color stable muscle. After an incubation time of 8 h, TBA-RS accumulation in PM was twice that in LL muscle. After 24 h of incubation, this difference is similar to that observed after 8 h. Under similar conditions, but on a shorter time scale (Anton et al., 1992), TBA-reactive material was more important in PM muscle than in LL muscle.

Similar results are observed when microsomal membranes are extracted from meat stored 8 days (D8) at 4 °C (Figure 1b) and incubated under the same conditions. Accumulation of TBA-RS in microsomal membranes of PM muscle was greater after day 8 than after day 0 (100 nmol after 8 h of incubation for D0 membranes and about 200 nmol for D8 membranes). In LL muscle, the influence of meat storage time (D0/D8) on TBA-RS accumulation is very low.

These results could be partly explained by the fact that, from the two bovine muscles, there are some important differences in the phospholipid and fatty acid compositions of microsomal fractions (Table 1a,b). Table 1a shows the phospholipid composition of the microsomal fraction of each muscle. The two muscles have high levels of phosphatidylcholine and phosphatidylethano-

Table 1. Phospholipids Composition (a) and Fatty Acids Composition (b) in the Microsomal Membranes of the Two Studied Muscles[a]

a. Phospholipids Composition

phospholipids	Longissimus lumborum	Psoas major
cardiolipids	3.8 ± 1.8[a]	6.8 ± 2.3[a]
phosphatidylethanolamine	26.1 ± 1.0[a]	34.3 ± 4.7[b]
phos. inositol + phos. serine	7.5 ± 1.3[a]	5.9 ± 1.4[a]
phosphatidylcholine	60.9 ± 0.6[a]	51.6 ± 5.4[b]
sphingomyeline	1.7 ± 1.2[a]	1.5 ± 1.5[a]

b. Fatty Acids Composition

fatty acids	Longissimus lumborum	Psoas major
saturated	33.1 ± 2.4[a]	29.9 ± 2.9[a]
monounsaturated	36.9 ± 5.0[a]	32.4 ± 4.4[a]
polyunsaturated	30.0 ± 5.8[a]	37.7 ± 6.5[a]

[a] Values, expressed in percentage ± SD of total content, within rows bearing different superscripts differ significantly at $P < 0.05$ (number of experiments = 4).

lamine. These two phospholipid fractions represent about 85% of the total phospholipids. PM has a significantly higher level of phosphatidylethanolamine than LL, and this component is more susceptible to peroxidation than the other phospholipid fractions (Pikul and Kummerow, 1991). Conversely, there is more phosphatidylcholine in LL than in PM muscle (Table 1a). These results agree with previous work (Genot et al., 1991). Cardiolipids probably arose from contamination by mitochondrial membranes (Daum, 1985). This contamination seems to be greater in PM muscle which is in agreement with the highest content of mitochondria in PM muscle when compared with LL tissue (Renerre, 1984). In these experimental conditions (Anton, 1993), cytochrome C oxidase activity measurements show a very low contamination of microsomes by mitochondria, according to the results of Albro et al. (1987) obtained from rat kidney microsomes.

Table 1b shows that the phospholipid fraction of PM muscle contains less saturated and monounsaturated but more polyunsaturated fatty acids, such as linoleic and arachidonic acids (results not shown), than the LL muscle; however these differences are not statistically significant. These results, obtained from microsomal preparations, are not very different from those obtained from whole muscle (Anton, 1993). As observed in fish (Luo and Hultin, 1986), it would be interesting to examine, in relation to the lipid peroxidation, whether or not there was a decrease in polyunsaturated fatty acids during meat storage. These polyunsaturated fatty acids react rapidly with oxidant radical species (as activated metmyoglobin but also free hydroxyl radicals and hydroperoxyl radicals) and, so, are more susceptible to the initiation and propagation of peroxidation.

Nevertheless, the differences in lipid peroxidation in relation to muscle type and time post-mortem are not exclusively due to the composition of the microsomal membranes. As noted in the litterature (Halliwell and Gutteridge, 1989), muscular cells contains many other pro- and antioxidants which can affect lipid peroxidation post-mortem.

Optical Spectroscopy. With a MetMb/H_2O_2 molar ratio of 1, the development of a characteristic spectra of ferrylmyoglobin ($P^{\bullet+}$-Fe^{IV}=O) is noticeable (curve 1 of Figure 2a) illustrating the decomposition of H_2O_2. This spectra is characterized by two maxima at 540 and 580 nm. No peak at 630 nm (characteristic of the protein in the iron(III) state) was observed. This result

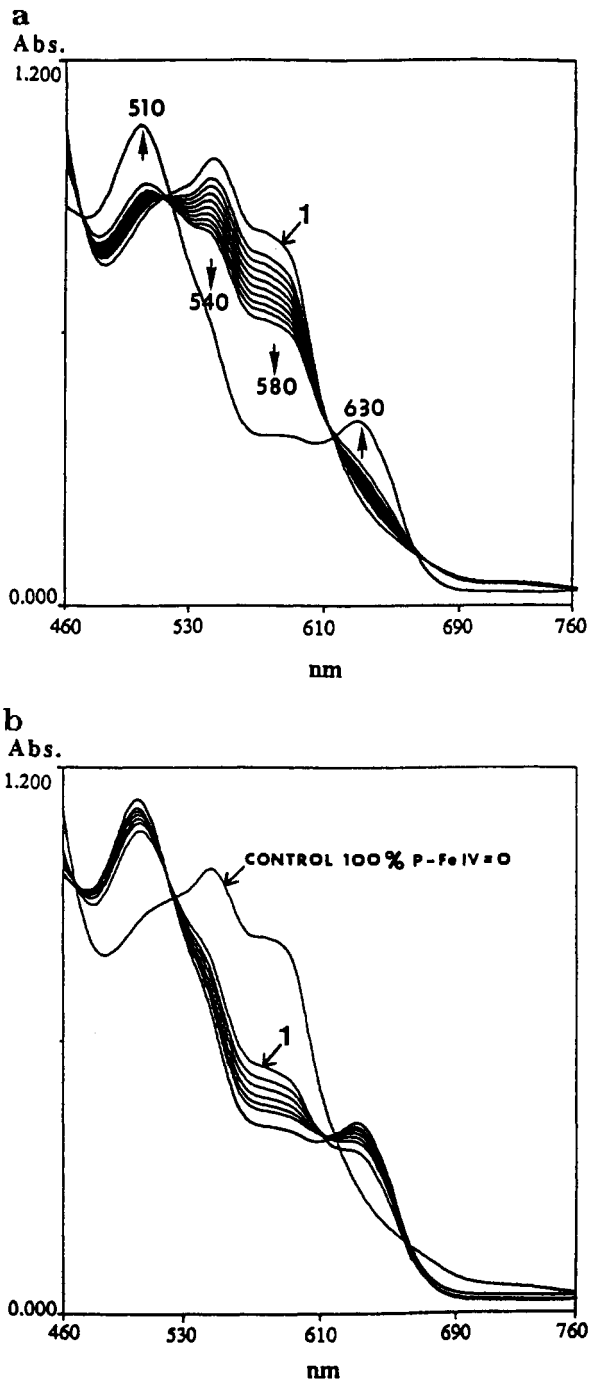

Figure 2. Visible spectra observed on reaction of metmyoglobin from Psoas major muscle (70 μM) with H_2O_2 (70 μM) (a) and in presence of day 0 microsomal membranes (0.21 mg/mL) (b). Spectra were recorded 30 s after mixing and then at 10 min intervals. The lower spectra corresponds to the 100% metmyoglobin recorded before H_2O_2 addition.

is similar to that obtained by Davies (1990) on myoglobin from horse heart. Tajima and Shikama (1993), with bovine heart muscle, reported peaks at 547 and 580 nm.

Nevertheless, this radical is not stable (Figure 2a): signals at 540 and 580 nm decrease slowly, while signals at 510 and 630 nm increase just after mixing and all during the experiment. This phenomenon, which illustrates the conversion of the ferryl state to the ferric state of iron, described many years ago, is poorly understood (Guilivi and Cadenas, 1993). In our experimental conditions, the half-life of the ferryl state was approximately 90 min and equivalent to a rate constant in the two muscles of approximately 0.007 min^{-1}.

Table 2. Characteristics of Ferrylmyoglobin Formation and Stability Measured by Optical Spectroscopy from Longissimus lumborum (LL) and Psoas major (PM) Muscles at Day 0 (D0) and Day 8 (D8) Storage[a]

incubation	decay rate constant (min^{-1})	initial ferrlmyoglobin (%)
MetMb LL + H_2O_2	0.0075 ± 0.0015[a]	100.0 ± 0.0[a]
MetMb PM + H_2O_2	0.0073 ± 0.0008[a]	100.0 ± 0.0[a]
MetMb LL + microsomes D0 + H_2O_2	0.0405 ± 0.0104[b]	32.8 ± 7.6[b]
MetMb PM + microsomes D0 + H_2O_2	0.0448 ± 0.0096[b]	36.9 ± 6.4[b]
MetMb LL + microsomes D8 + H_2O_2	0.0449 ± 0.0132[b]	36.4 ± 7.1[b]
MetMb PM + microsomes D8 + H_2O_2	0.0423 ± 0.0102[b]	33.6 ± 6.2[b]

[a] Values ± SD within columns bearing different superscripts differ significantly at $P < 0.05$ (number of experiments = 6).

These results agree with those of Tajima and Shikama (1993) who showed that this "autoreduction" was a function of pH. For these authors, the reaction was first order with a rate constant of $k = 1.06 \times 10^{-2}\ h^{-1}$ at pH 8.5 and at pH 7.0 this value would be approximately 0.0066 min^{-1}.

When metmyoglobin was incubated with microsomal membranes, before H_2O_2 addition (curve 1 of Figure 2b), only 35% of the ferrylmyoglobin was formed (Table 2). Under these conditions and similar to those used without membrane addition, the decay rate constant of the radical was enhanced by a factor of 6 in both muscles (Table 2). Because of the heterogeneity of the microsomal membrane preparations, high standard deviations for decay rates were found (Table 2). As demonstrated by Newman et al. (1991), the addition of membranes to the metmyoglobin–H_2O_2 system reduces the formation of radicals. This decrease in the formation of radical compounds comes from the microsomal membranes which can donate an electron from a specific site on the polyunsaturated fatty acyl lipid chain, initiating lipid peroxidation. The addition of microsomal membranes, extracted after an 8 day storage period, does not increase the decay rate constant of the radical form (Table 2). Moreover, no heme iron release (results not shown), using a modification of the Soret band at 410 nm, is observed with a H_2O_2/metmyoglobin ratio of 1. This result is in good agreement with that of Rice-Evans et al. (1989) who observed no iron release under the same conditions after 1 h and only 3.2% release after 3 h.

Although after incubation of microsomes in a mixture of H_2O_2 and MetMb, lipid peroxidation assessed by TBA-RS test is more important (Figure 1a,b) in the more color-labile muscle (PM), no statistical difference has been observed in the amount of activated metmyoglobin which is formed and in the stability of this protein radical (Table 2). These results show that activated-metmyoglobin was probably not the only agent of initiation of membranal lipid peroxidation in our system. In situ, it cannot be excluded that damage to the heme, which is in greater quantity in PM than in LL muscle (Anton, 1993), can induce iron release which could react with peroxides to give radicals such as HO• (from H_2O_2) or RO• and ROO• (from lipid hydroperoxides) (Kanner, 1992). With an H_2O_2/metmyoglobin ratio of 1/1, no iron release has been observed at room temperature, but at 37 °C (temperature of the peroxidation experiment) and after an incubation time of 24 h, such a release can take place and could induce differences observed in lipid peroxidation between the two muscles. Recently, Guilivi and Cadenas (1993), using sperm whale and an H_2O_2/MetMb ratio of 10/1, concluded that the dimerization of myoglobin upon its oxidation by H_2O_2 due to the cross-linking of Tyr residues (103-151) occurred. In the absense of Tyr-151 in beef myoglobin (Kagen, 1973), no formation of dimers would be detectable after its oxidation by H_2O_2 (Wilks and Ortiz de Montenallo, 1992).

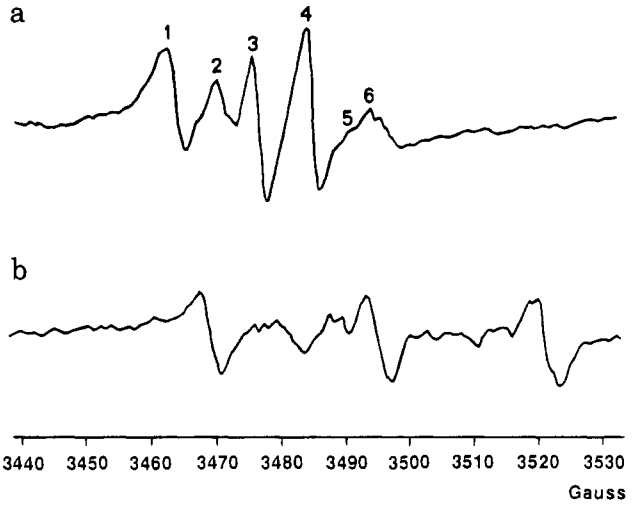

Figure 3. ESR spectra observed on reaction of metmyoglobin from Psoas major muscle (600 μM) with H_2O_2 (600 μM) in the presence of DMPO (0.1 M): without Trolox (a) and with Trolox (0.55 mM) (b).

ESR Spectroscopy. The interaction of hydrogen peroxide with metmyoglobin formed a myoglobin-derived radical which could be detected by ESR spectroscopy using the DMPO spin-trapping reagent.

Xu et al. (1990) have demonstrated that metmyoglobin and H_2O_2 alone did not produce any free radicals and that formation of radicals which were derived from the metmyoglobin–H_2O_2 reaction was too rapid to be detected by ESR spectroscopy without spin trapping.

The ESR signal of the spin trap adduct consists of six peaks (Figure 3a), due to hyperfine couplings, located at G = 3462, 3470, 3475, 3484, 3491, and 3495 respectively. The most pronounced peak in the spectrum was at 3484 G. This signal was identical to those observed under the same conditions by Xu et al. (1990) and Davies (1991) and can be attributed to a tyrosine peroxyl radical. This tyrosine peroxyl radical which can initiate oxidative damage to membranes, at position 103, would be formed by the reaction of oxygen with the tyrosine phenoxyl radical (Tew and Ortiz de Montellano, 1988; Davies, 1991; Newman et al., 1991; Guilivi et al., 1992). However, Kelman et al. (1994) indicated that the myoglobin-derived radical formed by reaction of metmyoglobin with hydrogen peroxide is not a tyrosine-based peroxyl radical.

This signal is rapidly formed and the spin-trap adduct (DMPO–tyrosyl radical) concentration is maximum after 2 min of reaction at pH 7. However, the spin-trap adduct also decayed rapidly (Figure 4) and the decay rate constant was 0.023 min^{-1} (Table 3). Between the two muscles, no differences can be observed in the amount of formed radical and in the decay rate constant (Table 3).

The effect of Trolox, at two different concentrations, on protein radical formation was examined. Trolox is the water-soluble form of α-tocopherol which acts as an antioxidant by a spin-trapping mechanism. Addition of Trolox (0.55 mM) to metmyoglobin solution, before

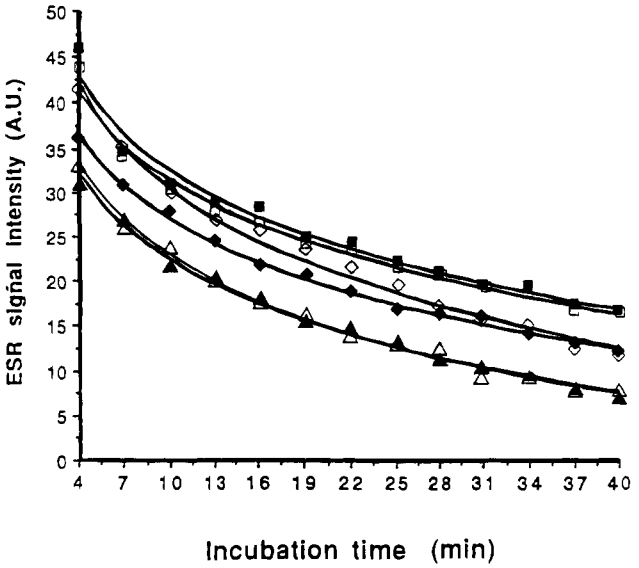

Figure 4. Kinetic of the ESR signal decay as measured by the 3484 G line intensity: (■) metmyoglobin from Psoas major muscle + H_2O_2; (□) metmyoglobin from Longissimus lumborum muscle + H_2O_2; (◆) metmyoglobin from Psoas major muscle + day 0 microsomes + H_2O_2; (◇) metmyoglobin from Longissimus lumborum muscle + day 0 microsomes + H_2O_2; (▲) metmyoglobin from Psoas major muscle + day 8 microsomes + H_2O_2; (△) metmyoglobin from Longissimus lumborum muscle + day 8 microsomes + H_2O_2.

Table 3. Characteristics of DMPO–Tyrosyl Radical Formation and Stability Measured by ESR Spectroscopy from Longissimus lumborum (LL) and Psoas major (PM) Muscles at Day 0 (D0) and Day 8 (D8) Storage[a]

incubation	decay rate constant (min^{-1})	3484 G line intensity at 4 min
MetMb LL + H_2O_2	0.0234 ± 0.0032^{a}	43.5 ± 8.5^{abd}
MetMb PM + H_2O_2	0.0233 ± 0.0036^{a}	45.5 ± 1.5^{ab}
MetMb LL + microsomes D0 + H_2O_2	0.0353 ± 0.0090^{ab}	41.7 ± 1.2^{abc}
MetMb PM + microsomes D0 + H_2O_2	0.0284 ± 0.0080^{ab}	35.7 ± 9.5^{bcd}
MetMb LL + microsomes D8 + H_2O_2	0.0413 ± 0.0089^{b}	33.2 ± 6.8^{cd}
MetMb PM + microsomes D8 + H_2O_2	0.0412 ± 0.0105^{b}	31.3 ± 5.6^{cd}

[a] Values ± SD within columns bearing different superscript differ significantly at $P < 0.05$ (number of experiments = 3).

adding H_2O_2, inhibits the formation of the tyrosine peroxyl ESR signal and enhances the concomitant production of a new ESR signal corresponding to the Trolox free radical compound (Figure 3b). The interaction of Trolox with ferrimyoglobin forms a quinone end product (Giulivi et al., 1992). No changes were observed in the Trolox ESR signal during a measurement time of 60 min indicating that this radical is more stable than the protein radical. The fact that the protein radical is completely inactivated by Trolox shows the importance of vitamin E as a protein antioxidant. With a concentration of Trolox of 5.5 mM, a disappearance of the Trolox free radical signal (results not shown) was observed. Giulivi and Cadenas (1993) indicated that Trolox inhibited dimerization and polymerization of sperm whale myoglobin. This point must be underscored because some reports have showed that the addition of vitamin E to diets of animals, by stabilizing the membrane lipids against peroxidation, improves both lipid and pigment stability in meats from different species (Buckley and Morrissey, 1992).

The addition of microsomal membranes to metmyoglobin before adding H_2O_2, decreases the amplitude of the ESR signal and increases the decay rate constant (Figure 4 and Table 3) by an average factor of 1.4 at D0 post-mortem. Moreover, although the differences are not significant, it appears that the addition of the microsomal fraction, extracted after an 8 day storage period, increased (1.3-fold) the decay rate constant of the tyrosine peroxyl radical more than the addition of the microsomal fraction extracted at day 0. As observed previously with optical spectroscopy, no statistical difference can be observed between PM and LL microsomes in the amplitude and stability of the ESR signal. Davies (1990) showed that the addition of linoleic acid reduced the intensity of the signal to about 90% of the control value.

During extraction, microsomal membranes may be also contaminated with peroxisomes and present catalase activity which can confuse experimental results as previously reported by Halliwell and Gutteridge (1989).

To better understand these oxidative processes which affect lipid peroxidation and myoglobin oxidation, further work on these radical reactions is needed.

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