about 2-fold with the addition of exogenous catalase. These results indicate that most of the formate oxidation is via the H_2O_2/catalase complex (compound I) and not by HO· radicals.

Most recently, we found that H_2O_2 activates metmyoglobin, probably to a porphyrin cation radical which initiates lipid peroxidation (Harel and Kanner, 1985). The oxidation of unsaturated fatty acids in muscle foods is one of the major causes of quality deterioration. It seems possible that endogenous generation of H_2O_2 could play an important role in the formation of the primary pool of radicals which initiate lipid peroxidation in muscle tissues.

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Registry No. H_2O_2, 7722-84-1.

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Muscle Membranal Lipid Peroxidation Initiated by H_2O_2-Activated Metmyoglobin

Stella Harel and Joseph Kanner*

Metmyoglobin (MetMb), H_2O_2-activated by glucose-oxidase/glucose system, initiated membranal lipid peroxidation. No such peroxidation occurred in the presence of the glucose oxidase system, H_2O_2, or MetMb alone. Heated MetMb maintained its capacity to be activated by H_2O_2. The accumulation of thiobarbituric reactive substances (TBA-RS) and oxygen absorption showed a higher rate of lipid peroxidation by H_2O_2-activated MetMb in microsomes separated from turkey than from chicken muscle tissues. Membranal lipid peroxidation initiated by activated MetMb was inhibited by low concentrations of either ascorbyl palmitate, α-tocopherol, or butylated hydroxytoluene (BHT). Inhibition was also observed by very low concentrations of ascorbic acid in the presence of EDTA. Only very high concentration of EDTA (1–10 mM) inhibited significantly membranal lipid peroxidation by activated metmyoglobin.

INTRODUCTION

The problem of lipid peroxidation in food systems is of significant importance in maintaining a stable food supply.

With modern techniques of food formulation and the production of structured food products, the problem of lipid peroxidation has increased in importance (Pearson et al., 1983). The use of mechanically deboned muscle tissues in the production of many of the restructured food products offers an entirely new approach to the development of food products. However, minced tissue is more readily subject to lipid peroxidation changes, either due to the disruption of the normal structure of the cells, or to the incorporation of more oxygen in the tissues.

Oxygen is vital for many purposes in biological systems and to the process of lipid peroxidation. The use of oxygen by biological matter is by controlled reactions. However, following food processing, such reactions can no longer be controlled. The electronic structure of oxygen, which reflects the Pauli principle, has two unpaired electrons at the energy level of π antibonding in triplet state $^3\Delta g$. The reaction of oxygen, therefore, is spin forbidden, with ground-state molecules of singlet multiplicity, such as polyunsaturated fatty acids (PUFA) or other molecules, in the same state. This barrier does not apply to reactions with atoms or molecules containing unpaired electrons, such as transition metals and free radicals. With regard

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to lipid peroxidation, one of the most important questions concerns the source of the primary catalysts that initiate peroxidation in the muscle food in vivo.

$$LH \xrightarrow{\text{catalyst}} L\cdot \quad (1)$$

Muscle tissue was found to generate hydrogen peroxide (Harel and Kanner, 1985), which seems to play an important role in the formation of the primary pool of catalysts for the initiation of lipid peroxidation. Hydrogen peroxide is the precursor of hydroxyl radicals (McCord and Day, 1978; Halliwell, 1978) and also an activator of iron heme proteins and enzymes (Shiga and Imaizumi, 1975; Hayashi and Yamazaki, 1979). Recently, Kanner and Kinsella (1983a) have shown that leukocytes which produce large amounts of superoxide radical (O_2^-) and hydrogen peroxide may be a focus for the initiation of lipid peroxidation in muscle tissues, and especially by peroxidases which produce chlorine and iodine radicals (Cl·, I·) in the presence of H_2O_2 and halides (Kanner and Kinsella, 1983b).

Iron heme proteins, and particularly myoglobin, are abundant in muscle tissues (Livingston and Brown, 1981). The ability of hematin and hemeproteins (myoglobin, hemoglobin, cytochrome C, peroxidases) to promote lipid peroxidation was demonstrated by many researchers (Hourowitz et al., 1941; Tappel, 1953; Kendrick and Watts, 1969; Kaschnitz, and Hatefi, 1975; Kanner et al., 1977a). The postulated mechanism of lipid peroxidation by heme compounds are based on homolytic scission of preformed fatty acid hydroperoxides to free radicals (Tappel, 1962; Kuhn et al., 1981). Heme proteins so far are catalyzers of the propagation step and not truly initiators of lipid peroxidation.

More recently, it was first discovered by us that the interaction of hydrogen peroxide with metmyoglobin (MetMb) or methemoglobin (Methb) led very rapidly to the generation of active species which promote membranal lipid peroxidation (Kanner and Harel, 1985). Activated MetMb could be described as true initiators of lipid peroxidation.

The present study was conducted to broaden our knowledge on activated MetMb and the initiation of lipid peroxidation in membranes separated from muscle tissues.

MATERIALS AND METHODS

Materials. Hydrogen peroxide (30% for synthesis) and trichloroacetic acid were purchased from Merck (Darmstadt, West Germany). Myoglobin type I from equine skeletal muscle, glucose oxidase type II, catalase free of thymol, potassium chloride, L-histidine free base, thiobarbituric acid, α-tocopherol, and serum bovine albumin were obtained from Sigma Chemical Co. (St. Louis, MO). Ethylenediaminetetraacetic acid disodium salt (EDTA), ascorbic acid, ascorbyl palmitate, and butylated hydroxytoluene were purchased from BDH Chemicals Ltd. (Poole, England).

Methods. Isolation of the microsomal fraction from muscle tissues was done by a procedure described previously (Apgar and Hultin, 1982; Kanner and Harel, 1985), protein determinations were conducted by the modified Lowry procedure (Markwell et al., 1978) by using BSA as standard.

Microsomes for the lipid peroxidation assay were incubated in a shaking water bath at 37 °C. The reaction mixture contained 1 mg of microsomal proteins per mL, the MetMb/H_2O_2 reactive compound, and 0.12 M KCl, 5 mM histidine buffer, pH 7.0. The reactions were initiated by activated MetMb with H_2O_2 (30 μM) or by glucose oxidase system (GOxS), which contained glucose oxidase, 0.04 units, and glucose, 400 μM.

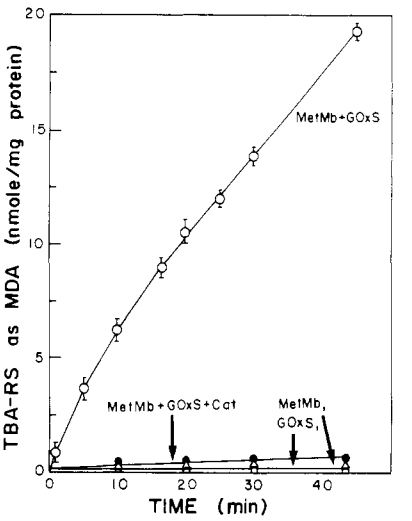

Figure 1. Effect of glucose oxidase system (GOxS) on membranal lipid peroxidation by activated MetMb. The reaction mixture contained glucose oxidase, 0.04 units, glucose, 400 μM, microsomes, 1 mg protein, KCl (0.12 M), EDTA (0.1 mM), and MetMb (30 μM) in 1 mL of histidine buffer (5mM), pH 7.3, at 37 °C. Error bars denote standard deviation (n = 3): ○, MetMb + GOxS; ●, MetMb + catalase (40 units) and GOxS; ΔΔ, MetMb or GOxS alone.

Thiobarbituric acid reactive substances were determined by the procedure of Bidlack et al. (1973), with the results reported as nmole of malondialdehyde (MDA) per mg of protein with a molar extinction coefficient of $E_{532} = 1.56 \times 10^5$ M^{-1} cm^{-1}.

Oxygen absorption during lipid peroxidation—in a reaction system containing activated MetMb, buffer, and microsomes as for MDA—was determined using an oxygen monitor (Yellow Spring Inst. Co., Model 53) with a Clark electrode.

The results are means of triplicates of one microsomal preparation; in the figures, each error bar (I) denotes the standard deviation.

RESULTS

The glucose oxidase system was adjusted for H_2O_2 generation of 1 nmol/mL/min (results not shown). Initiation of membranal lipid peroxidation occurred only in the presence of both the glucose oxidase system and MetMb, but not with only one of either compounds. Catalase (40 units) very effectively inhibited the activation of MetMb and the initiation of lipid peroxidation (Figure 1). Lipid peroxidation was initiated also by activation of MetMb (30 μM) with H_2O_2 (30 μM) for 3 min before the addition to the membranal suspension.

Lipid peroxidation of turkey and chicken (dark and light) muscle microsomes, initiated by activated MetMb, is shown in Figures 2 and 3. Thiobarbituric acid reactive substances accumulated and oxygen was absorbed at higher rates by the turkey than by the chicken muscle. Dark muscle microsomal lipids peroxidized faster than light muscle microsomal lipids. Heated MetMb maintained its capacity to be activated by H_2O_2 (Table I).

Activated MetMb dependent lipid peroxidation was inhibited by several antioxidants. Ascorbic acid at a low concentration enhanced lipid peroxidation but inhibited at a very high concentration.

The prooxidative effect of ascorbic acid was eliminated by the addition of a very low concentration of EDTA (Figure 4). In the presence of 10 μM EDTA, 10 μM ascorbic acid inhibited the initiation of lipid peroxidation

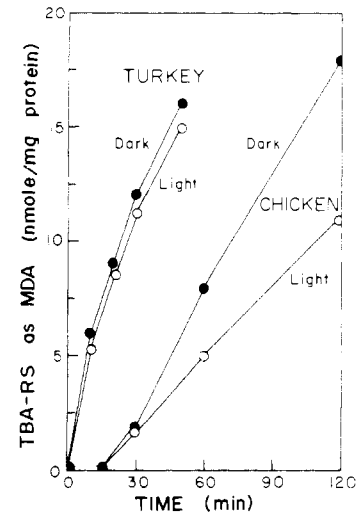

Figure 2. Turkey and chicken (from dark or light muscles) microsomal lipid peroxidation initiated by activated MetMb/H_2O_2 (30 μM each). The reaction mixture contained microsomes, 1 mg protein, KCl (0.12 M), and EDTA (0.1 mM) in 1 mL of histidine buffer (5 mM), pH 7.3, and was incubated at 37 °C.

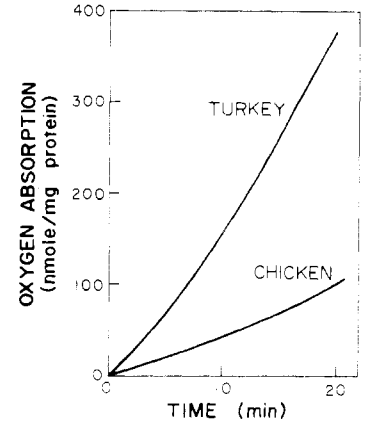

Figure 3. Oxygen absorption during membranal lipid peroxidation by activated MetMb/H_2O_2 (30 μM each). The reaction mixture (see Figure 2) was incubated at 37 °C.

Table I. Effect of Heating on Membranal Lipid Peroxidation Initiated by Activated MetMb[a]

treatment	TBA-RS as MDA, nmol/30 min	activity, %
control	10.8	100
MetMb heated for 1 min	9.2	85
MetMb heated for 3 min	8.6	80
MetMb heated for 5 min	8.6	80

[a] MetMb (30 μM) heated for several minutes at 98 °C and cooled before the interaction with H_2O_2 (30 μM) for 3 min.

by activated MetMb. Activated MetMb dependent lipid peroxidation was not inhibited by a low concentration of EDTA (10–100 μM), but 50% inhibition was obtained with 10 mM EDTA (Figure 5).

Membranal lipid peroxidation initiated by activated MetMb was partially inhibited by a very low concentration of either ascorbyl palmitate, α-tocopherol, or BHT (Table II) and completely inhibited by all antioxidants at a concentration of 15 μM.

DISCUSSION

Peroxidation of PUFA is a naturally occurring free-radical chain reaction that has been implicated as a mechanism of tissue damage (Pryor, 1980). The exact nature of the predominant forms of iron catalysts in muscle tissues which are involved in the initiation of lipid peroxidation in situ is lacking (Love, 1983). Scientists often refer to added metal salts and their complexes as "initiators" of lipid peroxidation. However, in most cases, the salts cause the decomposition of preformed lipid hydroperoxides. All commercially available unsaturated fatty acids contain traces of hydroperoxides (Halliwell and Gutteridge, 1984), thus most of the reports on the oxidation of lipids in model muscle foods studied the propagation stage. Nonenzymic catalysts for the propagation of lipid oxidation in muscle tissues have been studied and reveiwed by many researchers (Pearson et al., 1977; Love, 1983; Khayat and Schwall, 1983). Hultin and co-workers (Lin and Hultin, 1976; Shewfelt and Hultin, 1983) developed a muscle model for the determination of lipid

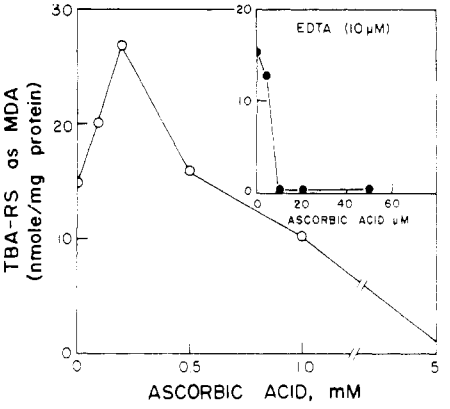

Figure 4. Membranal lipid peroxidation initiated by activated MetMb/H_2O_2 (30 μM each) as affected by ascorbic acid (○) and ascorbic acid in the presence of EDTA (10 μM) (●). The reaction mixture (see Figure 2) was incubated at 37 °C.

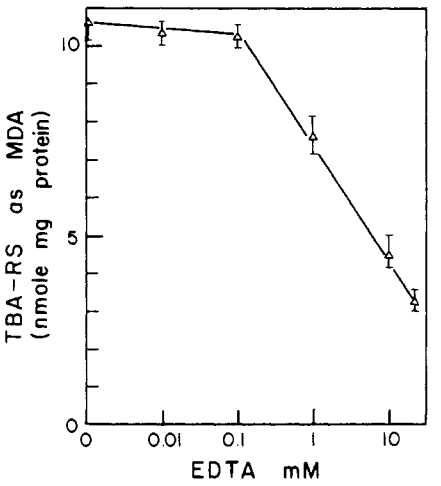

Figure 5. Membranal lipid peroxidation by activated MetMb/H_2O_2 (30 μM each) as affected by EDTA concentration. The reaction mixture (see Figure 2) was incubated at 37 °C. Error bars denote standard deviation (n = 3).

Table II. Effect of Several Antioxidants on Membranal Lipid Peroxidation Initiated by Activated MetMb[a]

treatment		TBA-RS as MDA, nmol/30 min	inhibition, %
control		11.5	0
BHT	5 μM	8.0	30
ascorbyl palmitate	5 μM	6.9	40
α-tocopherol	5 μM	4.0	65
each antioxidant	15 μM	0	100

[a] MetMb/H_2O_2 (30 μM each) preincubated for 3 min before the addition to microsomes (1 mg/mL of protein) in a 1-mL buffer of histidine, pH 7.3, at 37 °C, which contained 0.86 M ethanol. The antioxidants were solubilized in ethanol to a final concentration in the mixture reaction of 0.86 M ethanol.

peroxidation initiated by an enzymic microsomal NAD(P)H dependent system, in which lipid peroxidation was initiated by iron salts. Our muscle model system for the initiation of lipid peroxidation contains endogenous compounds found in muscle tissues, such as membranal lipids, myoglobin, and H_2O_2.

Hemoglobin, and especially myoglobin, are very abundant in muscle tissues (Livingston and Brown, 1981) and play essential roles in maintaining aerobic metabolism. During cutting, chopping, or grinding, oxyhemoglobin and oxymyoglobin are oxidized. The autooxidation of oxyhemoglobin and oxymyoglobin leads to the formation of MetMb or MetHb and O_2^-, which dismutate to H_2O_2 (Brown and Mebine, 1969; Misra and Fridovich, 1972; Satoh and Shikama, 1981; Wallace et al., 1982). Oxymyoglobin, which generates H_2O_2 during autooxidation, could activate its own molecule; however, endogenous H_2O_2 could be generated in muscle tissues also by other systems (Harel and Kanner, 1985).

The interaction of MetMb with H_2O_2 was reported to produce a free radical (King and Winfield, 1963) and later confirmed (Yonetani and Schleyer, 1967; Shiga and Imaizumi, 1975). This compound was found to oxidize a series of phenols (Shiga and Imaizumi, 1975) and uric acid (Ames et al., 1981) and to cause protein cross-linking (Rice et al., 1983).

The glucose oxidase system demonstrated that small amounts of H_2O_2, generated continuously, could activate MetMb and lipid peroxidation more efficiently than incubation of large amounts of H_2O_2 with MetMb. Activated MetMb species were not affected by catalase, but catalase could prevent their formation (Kanner and Harel, 1985).

The results demonstrated that the interaction of MetMb with H_2O_2 generated a new catalyst, unaffected by catalase. Lipid peroxidation initiated by activated MetMb was found to be dependent on pH, showing maximal activity similar to that of the pH in situ of muscle tissues. It was also found that the activity of the prooxidant decreased during the first minutes, but 50% of its activity remained stable for more than 30 min. Small amounts of H_2O_2 (1–10 μM) could activate MetMb for significant lipid peroxidation, however high concentrations of MetMb or methemoglobin inhibited the reaction (Kanner and Harel, 1985).

Lipid peroxidation of turkey muscle microsomes by activated MetMb was more rapid than that of chicken muscle microsomes. These differences were obtained by using both the accumulation of TBA-RS and oxygen absorption methods. The results in model system are closely correlated with those reported by others for muscle tissues (Wilson et al., 1976; Shagalovich and Kanner, 1981). From our findings, however, the reason for these differences is not clear (research on this topic is in progress).

As compared to the relative stability of fresh meats, cooked meats more readily develop off flavors (warmed-over flavor) (Pearson et al., 1977). Catalase, which is present in uncooked meat, might prevent partially the activation of metmyoglobin (Harel and Kanner, preceding paper), whereas, heated meat would have greatly reduced catalase.

Of great interest is the finding that heated MetMb retained its capacity to be activated by H_2O_2. This indicates that heating of muscle foods, which was shown to accelerate the production of H_2O_2 (Harel and Kanner, 1985), does not prevent the possible initiation of lipid peroxidation by activated MetMb.

The prevention and control of lipid peroxidation in muscle foods is an important goal for many industries. Ascorbic acid is a known antioxidant, but in the presence of metal ions it was shown to act also as prooxidant (Kanner et al., 1977b). Similar results were obtained by using ascorbic acid in muscle tissues (Deng et al., 1978).

In the presence of activated MetMb and microsomes, a low concentration of ascorbic acid accelerated lipid peroxidation and only a high concentration—5 mM—inhibited this reaction almost completely. Traces of iron (equivalent to 3–5 μM $FeCl_3$), which were found in our model system, could explain this behavior of ascorbic acid.

A low concentration of EDTA (10 μM) enhanced the antioxidative effect of ascorbic acid by 500-fold. Only at a very high (10 mM) concentration could EDTA alone inhibit lipid peroxidation initiated by activated MetMb. The antioxidative effect of EDTA seems to stem from two different mechanisms. The first arises from its metal chelating capacity, which inhibits metal "redox cycle" initiation of lipid peroxidation (Kanner, 1974, 1976; Searle and Willson, 1983). The second inhibitory activity of EDTA occurs at high concentration by its characteristics to act as a weak electron donor (Frisell et al., 1959; Holmstrom, 1964).

Several other antioxidants, such as ascorbyl palmitate, α-tocopherol, and BHT, at a concentration range of 1–10 μM inhibit initiation of lipid peroxidation by activated MetMb. This high efficiency of the antioxidants seems to derive from the ability of phenols and reducing compounds to act as electron donors, deactivating the catalyst, as well as suppressing lipid peroxy radicals and preventing propagation of lipid peroxidation.

When our data and those from the literature were used, a comprehensive reaction for initiation and autooxidation of lipids by myoglobin (heme proteins) has been drawn up (Kanner and Harel, 1985); these include the following: (a) Autooxidation and oxygen activation of oxymyoglobin to MetMb and H_2O_2. (b) Catalyst activation of MetMb by H_2O_2, like peroxidases, to porphyrin cation radical, P^+—Fe^{IV}=O. (c) Initiation of lipid peroxidation by P^+—Fe^{IV}=O via two-electron reduction of the catalyst, based on the results of uric acid oxidation (Howell and Wyngaarden, 1960) and titration of the catalyst with K_4 [Fe(CN)$_6$] (Kremer, 1981).

$$P^+—Fe^{IV}=O + LH \rightarrow P—Fe^{IV}=O + L\cdot + H^+ \quad (2)$$

$$L\cdot + O_2 \rightarrow LOO\cdot \quad (3)$$

$$LOO\cdot + LH \rightarrow LOOH + L\cdot \quad (4)$$

$$P—Fe^{IV}=O + LOOH \rightarrow P—Fe^{III} + LOO\cdot + {}^-OH \quad (5)$$

$$LOO\cdot + LH \rightarrow LOOH + L\cdot \quad (6)$$

(LH = unsaturated fatty acid; L· = allyl radical; LOO· = peroxyl radical; LOOH = hydroperoxide.) (d) Hydroperoxides which are generated during lipid peroxidation could also activate MetMb (Jones et al., 1977; Kalyanaraman et al., 1982). (e) Reducing compounds and the antioxidants deactivate the catalyst and inhibit lipid peroxidation by the following reactions:

$$P^+—Fe^{IV}=O + AH \rightarrow P—Fe^{IV}=O + A\cdot + H^+ \quad (7)$$

$$P—Fe^{IV}=O + AH \rightarrow P—Fe^{III} + A\cdot + {}^-OH \quad (8)$$

$$L\cdot; LO\cdot; LOO\cdot \xrightarrow{AH} LH; LOH; LOOH + A\cdot \quad (9)$$

$$L\cdot; LO\cdot; LOO\cdot \xrightarrow{A\cdot} LA; LOA; LOOA \quad (10)$$

(AH = hydrogen or electron donors.)

We assume that in muscle tissues one of the first steps leading to the initiation of lipid peroxidation is the gen-

eration of endogeneous H_2O_2 (Harel and Kanner, 1985). This could occur via the superoxide anion or directly and be the precursor of the first catalysts which appears to initiate lipid peroxidation—the hydroxyl radicals and activated MetMb (heme proteins).

Registry No. BHT, 128-37-0; H_2O_2, 7722-84-1; EDTA, 60-00-4; ascorbyl palmitate, 137-66-6; α-tocopherol, 59-02-9; ascorbic acid, 50-81-7.

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